Pharmacologic modulation of endometrial intracrinology and steroid receptor expression

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CONTENTS

Abstract

Steroid hormones, acting via their cognate receptors, are key players in fundamental reproductive events: implantation and endometrial bleeding. To understand local mechanisms regulating function and the effect of pharmacologic modulation it is essential to have an understanding of factors regulating ligand availability and steroid receptor expression in the physiological state and after pharmacologic manipulations.

This thesis encompasses studies analysing expression of steroid metabolising enzymes (intracrinology) and steroid receptor expression in human endometrium after three different pharmacologic manipulations.

1. *Endometrium exposed to a GnRH antagonist during controlled ovarian hyperstimulation*

   Mid-luteal phase endometrial biopsies were obtained from oocyte donors undergoing ovarian stimulation and from control women with regular periods. Immunohistochemistry and real-time quantitative polymerase chain reaction (QRT–PCR) were used to compare protein and mRNA expression of sex steroid receptors and steroid metabolising enzymes. Significant alterations in the expression of sex-steroid receptors and their metabolizing enzymes were demonstrated. These changes may lead to alterations in the activity and intracellular availability of estrogens, progestogens and androgens in endometrium of women treated with a GnRH antagonist during controlled ovarian hyperstimulation. Their impact on embryo implantation merits further evaluation.

2. *Endometrium exposed to hormone replacement therapy (HRT)*

   Endometrial biopsies from postmenopausal women not using HRT and from HRT users were collected during and outside unscheduled bleeding episodes. Immunohistochemical analysis of endometrial sex steroid receptors was performed and the relationship between expression and bleeding patterns studied. Despite the predominantly progestational effect of continuous combined HRT used in the study, the steroid receptor expression in the postmenopausal endometrium differed from that seen in the premenopausal secretory phase of menstrual cycle and after long-term

progestogen-only administration, suggesting that different local mechanisms are involved in HRT-related unscheduled bleeding.

3. *Endometrium exposed to intrauterine delivery of a progesterone receptor antagonist*

   Women were randomised to intrauterine administration of either the antigestogen, ZK230211 (ZK-IUS) or Levonorgestrel (LNG-IUS) prior to hysterectomy. Endometrium was obtained from hysterectomy specimens. Bleeding patterns, endometrial morphology and content of ZK230211 were evaluated. Expression of sex steroid receptors, proliferation markers; phosphorylated Histone 3 (PH3) and Ki-67, and Insulin-like Growth Factor-Binding Protein-1 (IGFBP-1) were evaluated by immunohistochemistry (IHC). Administration of the antigestogen demonstrated novel effects such as an absence of IGFBP-1 and increase in progesterone receptor expression. These results suggest that intrauterine administration of an antigestogen is feasible and trials need to be undertaken to test clinical efficacy.

These studies, in pre and postmenopausal women, demonstrate that endometrial sex steroid receptor expression and enzymes determining intracellular steroid (ligand) availability are modulated by exogenous steroid manipulation.

Declaration

Except where due acknowledgement is made by reference, the studies undertaken in this thesis were the work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

All immunohistochemical analyses except the one for Insulin-like Growth Factor–Binding Protein-1 (IGFBP-1) expression were conducted at the University of Edinburgh Centre for Reproductive Biology. The IGFBP-1 immunohistochemistry was conducted in the University of Turku, Turku, Finland.

Dr Susheel Vani

My contribution to the studies included in this thesis

Study 1: Mid-luteal endometrial intracrinology following controlled ovarian hyperstimulation and use of a gonadotrophin releasing hormone antagonist

In this study, I was involved in recruiting women, in collection of endometrial samples, processing of endometrial tissue for immunohistochemistry and RT-PCR studies. This was my first laboratory based study and hence with supervision from laboratory technicians, I conducted all immunohistochemistry (Immunohistochemistry for Progesterone Receptor (PR), Estrogen Receptor α (ERα), Estrogen Receptor β (ERβ), Androgen Receptor (AR), 3β-hydroxysteroid dehydrogenase enzyme (3βHSD) and 17β-hydroxysteroid dehydrogenase type 5 enzyme (17βHSD5) expression) and Taqman RT-PCR (PR mRNA, ERα mRNA, ERβ mRNA, AR mRNA, 3βHSD1 mRNA and 17βHSD2 mRNA) experiments. I double-scored the immunohistochemistry slides with one of the technicians in Professor Critchley's laboratory group. Once the data were gathered, I was responsible, with some help from colleagues, for the statistical analysis. Being the first author, I made the most significant contribution in writing the paper that was then published in Human Reproduction.

Study 2: Endometrial expression of steroid receptors in postmenopausal hormone therapy users: relationship to bleeding patterns

This study was part of a larger study of bleeding mechanisms in women on Hormone Replacement Therapy (HRT), conducted at the Menopause Clinic in King Edward Memorial Hospital, Perth, Western Australia between 2003 and 2005. As part of the larger study, Professor Martha Hickey's group in Australia had already examined a number of factors such as endometrial vascular density and perivascular support, uterine natural killer cells and matrix metalloproteinases and their tissue inhibitors in these endometrial samples. Steroid receptor expression had not been analysed and hence this collaborative study was initiated. Paraffin blocks of endometrial samples were received at the University of Edinburgh Centre for Reproductive Biology. I conducted all the immunohistochemistry (PR, AR, ERα, ERβ and Glucocorticoid Receptor (GR)) experiments and double-scored the slides.

Because of the nature of the patient groups, special statistical tests were required and hence help was sought from Dr Rob Elton, medical statistician. Once the results were available, being the first author, I made the most significant contribution in writing the paper that was then published in the Journal of Family Planning and Reproductive Health Care.

Study 3: Intrauterine release of progesterone antagonist ZK230211 is feasible and results in novel endometrial effects: a pilot study

This proof of concept study was a collaborative study between the Centre for Reproductive Biology, University of Edinburgh and the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital, Finland. To establish the feasibility of the use of a progesterone antagonist intrauterine system, data on bleeding patterns and histological data had already been gathered. However the effects on endometrial steroid receptor expression and on proliferation markers was yet to be established and hence this collaborative study was undertaken. Paraffin blocks of endometrial samples were received at the University of Edinburgh Centre for Reproductive Biology. I conducted the immunohistochemistry (PR, ERα, ERβ, AR, proliferation markers Ki-67 and phosphorylated Histone 3 (PH3)) experiments and double-scored the slides. The IGFBP-1 immunohistochemistry was conducted in the University of Turku, Turku, Finland. I was involved with the statistical analysis and once the results were available, I made a significant contribution (literature search, data analysis, report writing) in preparing the final report based on the studies conducted at the Centre for Reproductive Biology, University of Edinburgh. These data were subsequently prepared for publication. Although not the first author, my contribution to manuscript preparation and data analysis was substantial. The paper was published in Human Reproduction and I am the second author in this publication.

Acknowledgements

I write this vote of thanks with a deep sense of gratitude to my supervisor Professor Hilary Critchley. Right from the inception of my research work till the stage of completion, I have been motivated and inspired by her clarity of thought and guidance. I have no doubt that this immensely fruitful experience will have a significant positive impact on the rest of my career. I am profoundly grateful to her for all the support.

To someone with a predominantly clinical background, the lab bench and the shelves full of unknown chemicals can seem a daunting prospect. Thanks to the 'HODC lab group' members, my transition was very streamlined and indeed enjoyable. In particular, I wish to thank Morag Hamilton, Teresa Henderson, Lynsey Boswell, Anne Grant and Pamela Cornes. Colleagues in other research groups helped with relevant areas of their expertise and thanks are due to them for their help with my projects.

Ted Pinner deserves a special word of thanks for help with graphics included in research papers and presentations made in relation to the research studies.

Several collaborators have made significant contributions to the respective studies in this thesis. I would like to specially thank Professors J Ian Mason, Martha Hickey, Ian Fraser and Drs Oskari Heikinheimo and K J Thong for their valuable contributions and guidance.

Unwavering support from the family makes any work easier and enjoyable. I would like to extend my special thanks to my wife, Archana, and my son Shreenil, for being so patient and supportive. Continuous words of encouragement from my parents, has prodded me on and without their support and blessings; the task would have proved much more tedious. Those phone calls from India meant a lot to me and I am very grateful for their support.

Lastly, all the women who took part in the studies included in this thesis deserve a special word of thanks. Without their support this work would not have been possible and their help is gratefully acknowledged.

Abbreviations

AP-IUS	Anti Progestin – Intra Uterine System
AR	Androgen Receptor
BTB	Breakthrough Bleeding
COH	Controlled Ovarian Hyperstimulation
DAB	Diaminobenzidine
dH_20	Distilled Water
DMPA	Depo Medroxy Progesterone Acetate
EB	Endometrial Biopsy
EDTA	Ethylene Di-amino Tetra Acetate
ERα	Estrogen Receptor α
ERβ	Estrogen Receptor β
FSH	Follicle Stimulating Hormone
GnRHa	Gonadotrophin Releasing Hormone agonists
GnRHantag	Gonadotrophin Releasing Hormone antagonists
GR	Glucocorticoid Receptor
hCG	human Chorionic Gonadotrophin
3βHSD	3 beta hydroxysteroid dehydrogenase
17βHSD	17 beta hydroxysteroid dehydrogenase
HRT	Hormone Replacement Therapy
ICSI	Intra-Cytoplasmic Sperm Injection
IGFBP-1	Insulin-like Growth Factor-Binding Protein -1
IHC	Immunohistochemistry
IVF	In-Vitro Fertilisation
LH	Luteinizing Hormone
LMP	Last Menstrual Period
LNG-IUS	Levonorgestrel Intra-Uterine System
mIgG1	mouse Immunoglobulin G1
MMP's	Matrix Metalloproteinases
MRC	Medical Research Council
mRNA	messenger Ribose Nucleic Acid

NHS	Normal Horse Serum
OHSS	Ovarian Hyperstimulation Syndrome
OR	Oocyte Retrieval
PA-IUS	Progesterone Antagonist – Intra-Uterine System
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline Tween
pH3	phosphorylated Histone 3
POC	Progestogen-Only Contraception
PR	Progesterone Receptor
PRA	Progesterone Receptor A
PRB	Progesterone Receptor B
QRT-PCR	Quantitative Real Time – Polymerase Chain Reaction
TIMP's	Tissue Inhibitors of Matrix Metalloproteinases
uNK cells	Uterine Natural Killer cells
ZK-IUS	ZK230211 releasing – Intra Uterine System

Chapter 1

Literature Review

1.1 General Introduction

The endometrium is a target organ for steroid hormones such as estrogens and progesterones. These regulate the development and function of the human endometrium. There is an increasing appreciation of the fact that other steroid hormones such as androgens and glucocorticoids play important roles in the varied functions of the endometrium such as menstruation and repair and embryo implantation. It is also being recognised that the activity of the various steroid hormones is directly or indirectly responsible for endometrial pathology that is likely to be the underlying cause for a variety of clinical conditions *inter alia* infertility, breakthrough bleeding and recurrent miscarriages. This thesis mainly describes data relating to steroid receptor expression after pharmacological manipulation of the endometrium in the context of three clinical scenarios namely embryo implantation in assisted conception cycles, effects of postmenopausal hormone replacement therapy and use of a progesterone antagonist for the treatment of heavy menstrual bleeding. Through a summary of the published data and new results based on our studies, we hope to gain further understanding of the effects of steroid hormones and the modulation of steroid receptor expression on human endometrium.

For successful conception, embryo implantation requires the presence of an optimum state of endometrium. This optimum state is believed to occur during a short window of time in the mid-secretory phase; commonly described as the 'window of implantation'. The endometrial characteristics that are present in this crucial window are a result of a complex interplay of multiple factors. Because of extensive circumstantial evidence, there is little doubt in stating that the sex steroids and their receptor modulation play a central role in the processes involved leading up to the ideal conditions required for successful implantation. With the onset of IVF/ICSI treatments in the last three decades, an understanding of this topic has no longer remained an academic necessity but an urgent clinical need. A clear understanding and then a beneficial manipulation of the endometrium at this crucial time could, potentially, lead to improved pregnancy rates. Chapter 4 of this thesis details the current understanding of the endometrial intracrinology and its relationship to steroid receptor expression and, based on new data, proposes further

explanations of their role in embryo implantation in the context of use of Gonadotrophin Releasing Hormone antagonists (GnRHantag).

Menopause in women leads to wide ranging changes involving all body systems. Postmenopausal hormone replacement therapy (HRT) was, until recently, thought by some to be a panacea for all menopausal problems in women. Increasing experience with current HRT regimes has proved this to be far from the truth. An increasing awareness of the various side effects of HRT use has provoked a complete rethink on the role of HRT in managing menopause. Nonetheless, there is little doubt that in carefully selected women HRT use can significantly alleviate the menopausal vasomotor symptoms and provide other benefits such as bone protection and cardiovascular protection. However a significant proportion of women on HRT will suffer from breakthrough bleeding forcing them to stop the use of HRT. A clear understanding of the mechanisms involved in postmenopausal breakthrough bleeding could not only help us devise better treatment regimes but could also help solve a long standing biological and clinical conundrum – that of progesterone related breakthrough bleeding. Chapter 5 describes our current understanding of processes related to steroid hormone expression with a special focus on their relationship to postmenopausal breakthrough bleeding.

Among all gynaecological outpatient referrals, the commonest reason is for treatment of heavy menstrual bleeding and other menstrual disorders. Among the range of medical therapies developed, progestogens, plays a major part in treating menstrual disorders. Whilst oral progestogens have been shown to be less effective in reducing menstrual blood loss, the Levonorgestrel - releasing Intra Uterine System (LNG-IUS) has shown excellent results in treating heavy menstrual bleeding. Initially introduced as a contraceptive, currently, in Europe, more women use it for relief of menstrual disorders. However just like any progesterone derivative, breakthrough bleeding remains a clinically significant side effect. Progesterone antagonists (PA) have been known to cause amenorrhoea without the unwanted side effects of breakthrough bleeding. Whilst oral Mifepristone has been tested in humans, a Progesterone Antagonist – IUS (PA-IUS) has, till date, not been trialled. Animal studies have shown promising results with several PAs and for the first time, a proof of concept study using a PA-IUS was performed in

women suffering from menstrual disorders. Chapter 6 describes the background understanding of this emerging area of high clinical importance with a special emphasis on endometrial markers in the presence of a PA-IUS.

The literature review that follows will expand on the background and the published data relevant to these three clinical scenarios with a particular emphasis on endometrial steroid receptor modulation. It will also propose avenues of future research and possible clinical applications.

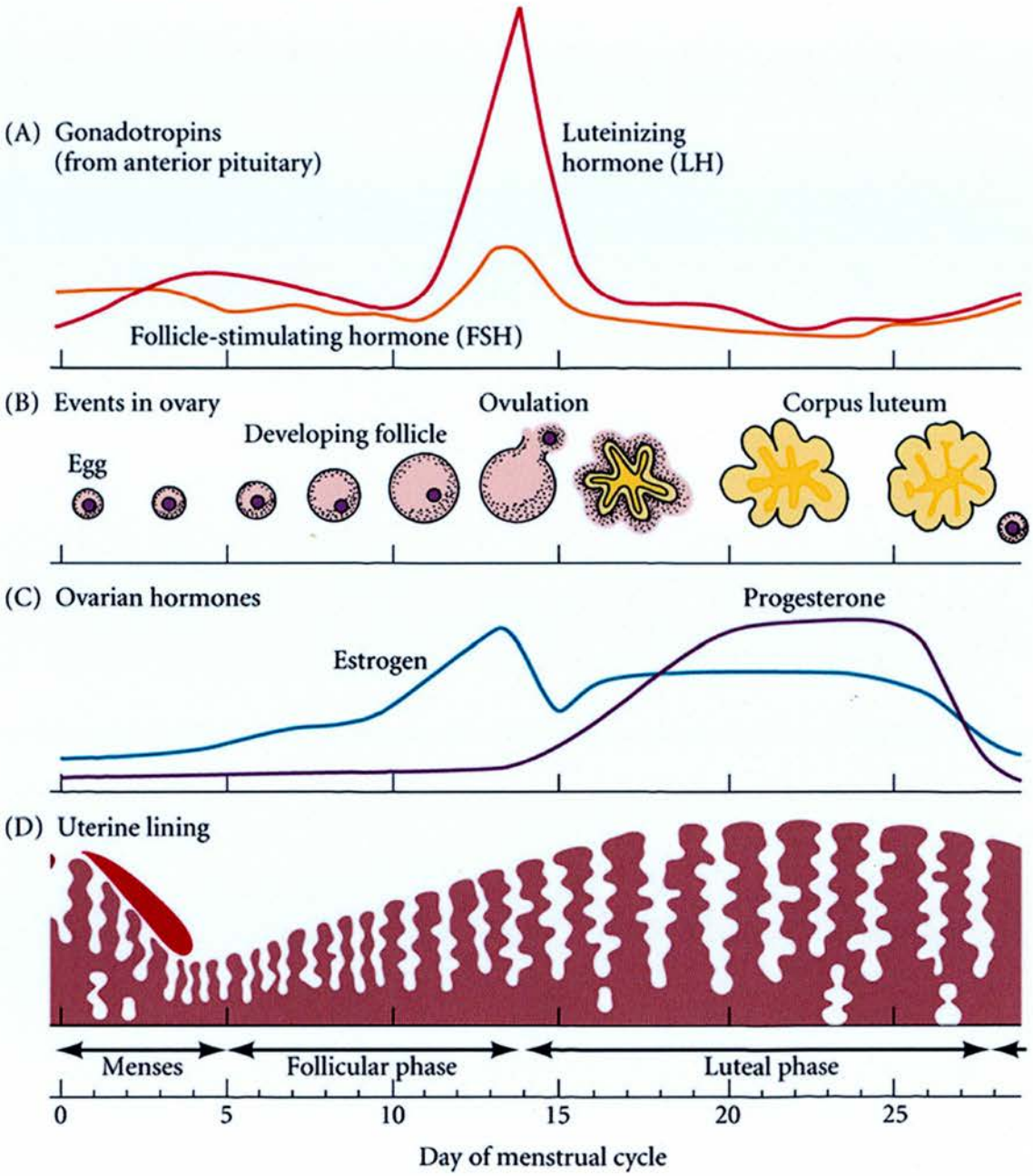
1.2 Hypothalamo-Pituitary-Ovarian axis

Neurons that produce the Gonadotrophin Releasing Hormone (GnRH) originate in the olfactory area and during embryogenesis migrate along cranial nerves to finally reside in the arcuate nucleus of the hypothalamus (Speroff and Fritz, 2005). GnRH is secreted in a pulsatile manner by these neurons from the hypothalamus. Feedback from target gland hormones, pituitary hormones and from GnRH itself, modify the release of further GnRH through an array of neurotransmitters including dopamine, norepinephrine, epinephrine, endorphin, serotonin and melatonin. Once released it is then transported through the hypophyseal portal circulation to reach the putative target sites on the gonadotrope cells in the anterior pituitary. GnRH has a half-life of 2-4 minutes. Because of this rapid degradation, combined with the enormous dilution on entry into the peripheral circulation, biologically effective amounts of GnRH do not escape the portal system. Therefore, control of the reproductive cycle depends on constant release of GnRH (Speroff and Fritz, 2005). GnRH acts on the gonadotrope cells of the pituitary. In the gonadotrope cells, GnRH binds to G protein coupled receptors and initiates a series of physiological events leading to the synthesis and release of gonadotrophins; Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) in a highly coordinated fashion (Ramakrishnappa *et al.*, 2005). The GnRH receptors are regulated by many agents including GnRH itself, inhibin, activin and sex steroids (Kaiser *et al.*, 1997). Binding of GnRH to its receptor in the pituitary activates multiple messengers and responses. The immediate response is the secretion of gonadotrophins while the delayed response is partly responsible for the large surge of gonadotrophins at midcycle. The delayed response, which ultimately leads to a surge in

gonadotrophins midcycle, requires estrogen exposure and can be augmented by progesterone (Speroff and Fritz, 2005). Both FSH and LH are released from the gonadotrope cells in a pulsatile manner into the systemic circulation. They in turn control the processes of gametogenesis and steroidogenesis in the ovary as detailed in section 1.2.2.

Negative feedback loops ensure control of further hormone secretions. Estrogen and high levels of progesterones inhibit GnRH pulses in the hypothalamus. In the proliferative phase, estradiol and inhibin – B suppress the release of FSH. In the luteal phase; progesterone, estradiol and inhibin-A act centrally to suppress gonadotrophins and new follicular growth (Speroff and Fritz, 2005) (Figures 1.1 (a) & (b)).

Figure 1.1(a) Schematic representation of the crucial events in the Hypothalamo-Pituitary-Ovarian (HPO) axis.



FSH – mIU/ml; LH – mIU/ml; Estrogen – pg/ml; Progesterone – ng/ml

Figure adapted from DevBio – A companion to Developmental Biology (2006) (8th Ed), by Scott F Gilbert

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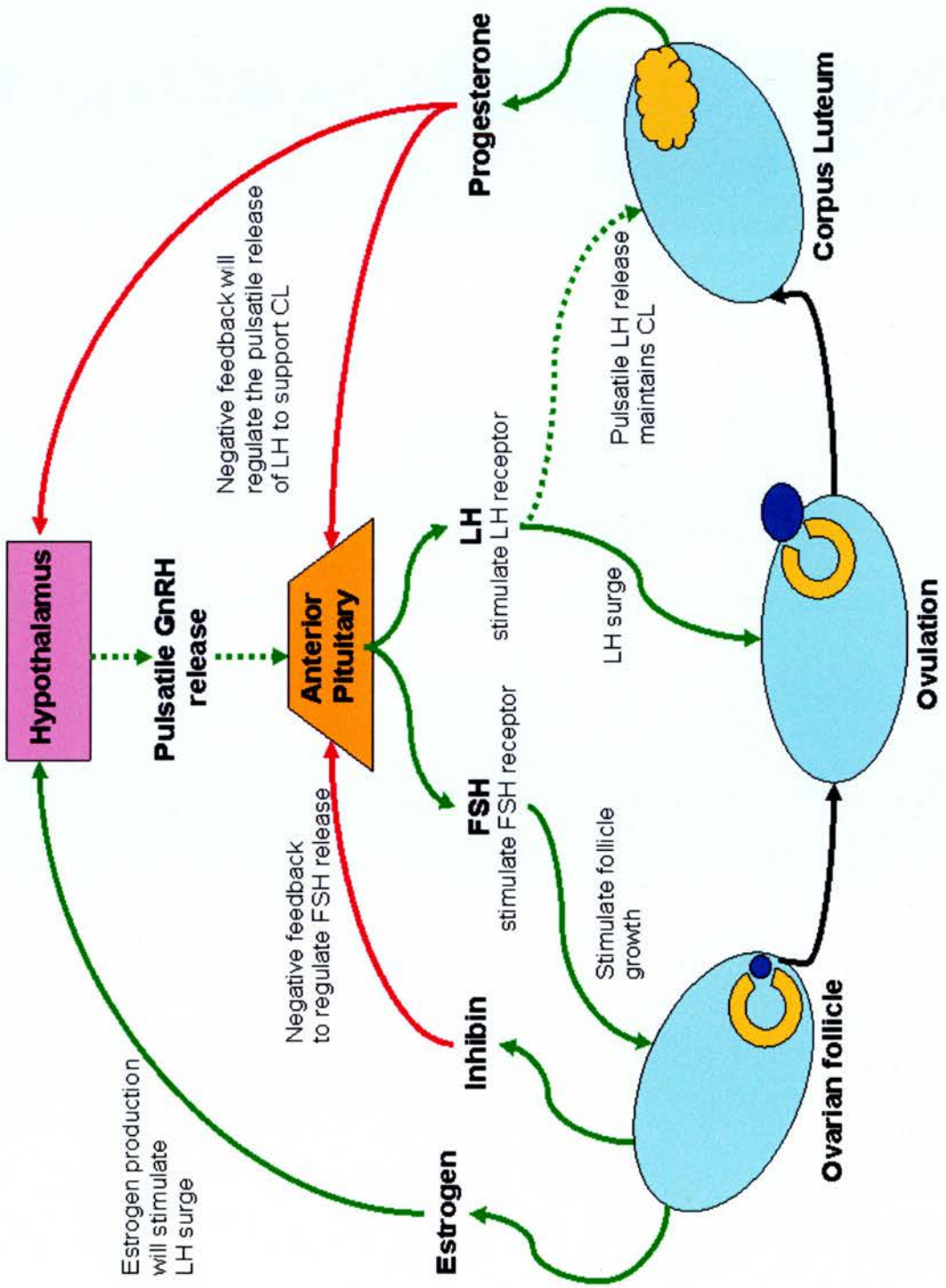
http://8e.devbio.com/image.php?id=542

Figure 1.1(b) Schematic representation of the regulation of the Hypothalamo-Pituitary-Ovarian (HPO) axis.



1.2.1 Role of Gonadotrophin Releasing Hormone

Human Gonadotrophin Releasing Hormone (GnRH) is a decapeptide with 10 amino acids. GnRH acts through the GnRH receptor. Three types of GnRH receptors have been identified in the vertebrates (Troskie *et al.*, 1998). GnRH receptor Type II is found in the humans and is widely expressed in the central nervous system and in reproductive tissues. It is also present in the gonadotropes in the anterior pituitary and hence it is believed that most of the GnRH mediated reproductive functions are modulated through this receptor type (Millar *et al.*, 2001). GnRH is also known to have an autocrine – paracrine function throughout the body.

In relation to the reproductive system, GnRH receptors have been found in several extra-pituitary sites like the ovary, breast, placenta, fallopian tubes and human endometrium (Ramakrishnappa *et al.*, 2005). Because of its widespread presence, it is believed that GnRH has a role to play in wide ranging clinical situations like reproductive cancers, infertility and potentially in male and female contraception.

The principal function of GnRH involves the stimulation of gonadotrope cells in the anterior pituitary leading to the secretion of FSH and LH. GnRH is known to stimulate the secretion of both FSH and LH, but there is ongoing debate regarding the exact mechanisms involved in the way one releasing hormone leads to the secretion of two gonadotrophins. One possible mechanism is related to the pulse frequencies of GnRH release. High GnRH pulse frequencies (one pulse every 30 minutes) favour LH synthesis whereas low GnRH pulse frequencies (one pulse every 120 minutes) favour FSH synthesis (Kaiser *et al.*, 1997). Whilst other mechanisms to explain this phenomenon have been proposed, it is interesting to note that a possibility of a separate FSH releasing factor has been postulated (Padmanabhan and McNeilly, 2001).

1.2.1.1 GnRH and Infertility

Pulsatile GnRH is used for ovulation induction in women with hypogonadotrophic hypogonadism. However it is the use of GnRH analogues, GnRH antagonists and gonadotrophins that has revolutionized the treatment protocols in assisted conception. Continuous stimulation with GnRH analogues leads to an initial flare

up followed by prolonged suppression of gonadotrophins and this in turn leads to a fall in levels of sex steroids.

Premature LH surges were responsible for the cancellation of a large number of In-vitro Fertilization (IVF)/Intracytoplasmic Sperm Injection (ICSI) treatment cycles. With the introduction of treatment strategies to prevent premature LH surges, the cycle cancellation rate has been significantly reduced and IVF/ICSI success rates have significantly improved (Hughes *et al.*, 1992). GnRH analogues (Hughes *et al.*, 1992) and more recently GnRH antagonists (Albano *et al.*, 2000) have been shown to reliably prevent premature LH surges and these have been increasingly employed to achieve this goal. As described later, in comparison to the use of GnRH analogues, there are several advantages of using GnRH antagonists for this purpose. However, with the use of GnRH antagonists, there are concerns of reduced pregnancy rates (Al-Inany *et al.*, 2006).

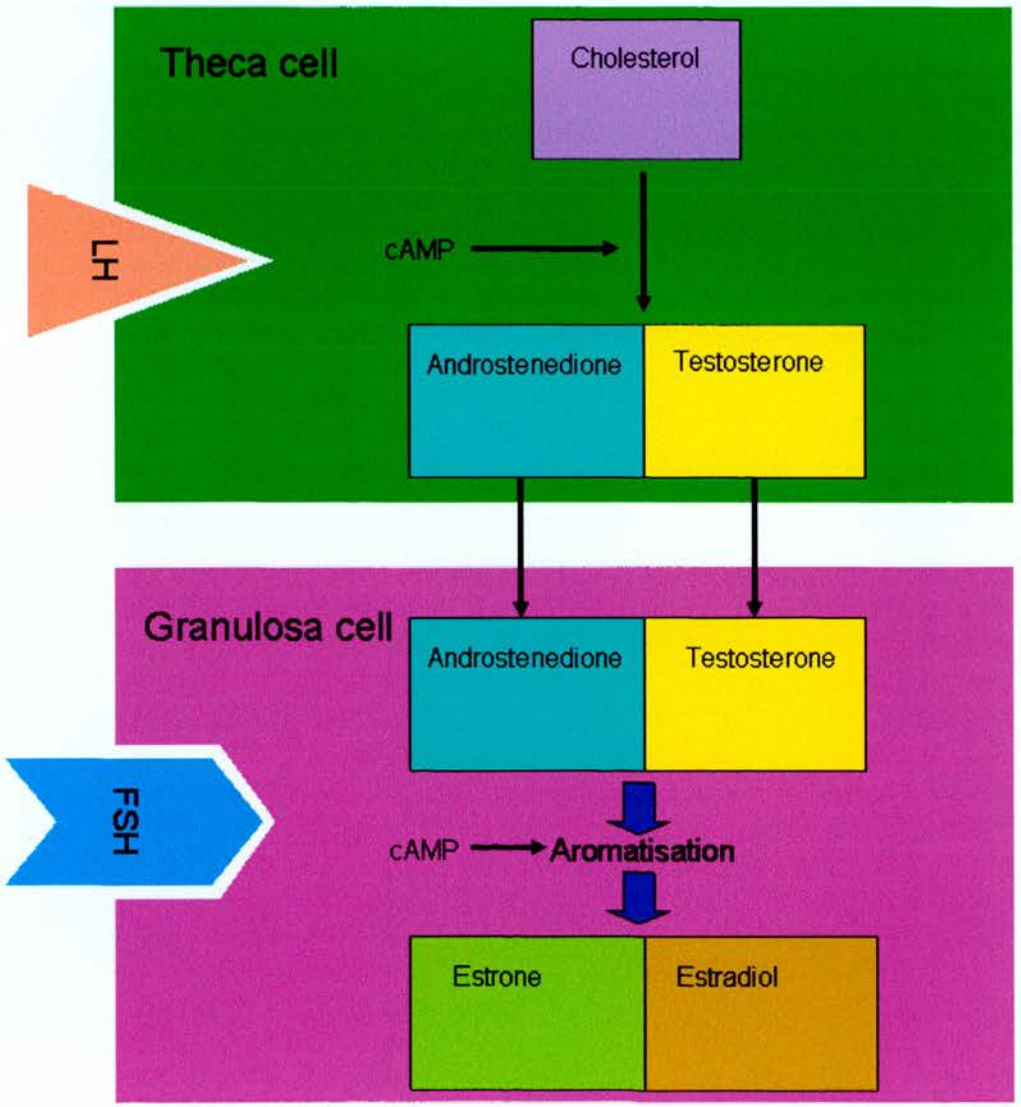
The clinical pregnancy rates in IVF/ICSI treatment cycles are approximately 25%. A recent meta-analysis comparing (calculated) live birth rates in GnRH agonist and GnRH antagonist treated cycles found a non-significant drop of 2.7% in the live birth rates in GnRH antagonist cycles. However the study authors did conclude that 'If the observed non-significant difference in live birth rates of 2.7% represents the true difference present in the population, it is likely that this will become significant ($P < 0.05$) with the addition of more comparative studies' (Kolibianakis *et al.*, 2006). The reasons for this putative drop in pregnancy rates are unclear. Whilst GnRH analogues and GnRH antagonists influence steroidogenesis through their suppression of gonadotrophin levels, it is possible that they have some direct effects through their actions on the GnRH receptor. Not surprisingly, a possible role of the GnRH receptor in embryo implantation related processes has been postulated (Casan *et al.*, 1998).

1.2.2 Role of Gonadotrophins

FSH and LH are hormones released from the gonadotrope cells of the anterior pituitary. In the human preantral and antral follicles, LH receptors are present only on the theca cells and FSH receptors only on the granulosa cells (Kobayashi *et al.*, 1990; Yamoto *et al.*, 1992). The sequence of events responsible for follicular

growth, maturation, ovulation and the development and regression of corpus luteum are complex. As per the widely accepted two-cell two-gonadotrophin hypothesis, FSH stimulates the granulosa cells in the developing follicle in the ovary and through aromatization of androgens, leads to the synthesis and secretion of estrogen (Figure 1.2). FSH stimulation propels the primordial follicle to the preantral stage. LH stimulates the theca cells in the ovarian follicle. The cholesterol within the theca cells is then converted to androgens. The androgens produced within the theca cells then diffuse into the granulosa cells where, through FSH-induced aromatization, they are converted to estrogens (Erickson, 1996; Kobayashi *et al.*, 1990). The FSH and LH activity is mediated through their respective receptors. In the preantral and antral follicle, FSH receptors are only present on the granulosa cells and LH receptors are only present in the theca cells (Kobayashi *et al.*, 1990; Yamoto *et al.*, 1992). As the granulosa cells respond to FSH, further proliferation and growth of the granulosa cells is associated with an increase in FSH receptors. With progressive maturation of the follicle, the preovulatory follicle produces increasing amounts of estrogen. During the late proliferative phase, estrogens rise rapidly, reaching a peak approximately 24-36 hours prior to ovulation (Pauerstein *et al.*, 1978). The onset of the pre-ovulatory LH surge occurs when the peak levels of estradiol are achieved (Fritz *et al.*, 1992). Ovulation is thought to occur about 34-36 hours after the onset of the LH surge and about 10-12 hours after the LH peak. Following ovulation, continued LH stimulation is responsible for the maintenance of the corpus luteum, with corpus luteum being the main source of progesterone.

Figure 1.2 Diagram illustrating the activity of the gonadotrophins on the ovarian follicle
(Adapted from Speroff and Fritz, 2005)



1.2.3 Controlled Ovarian Hyperstimulation

Although the first conception through IVF was in a natural cycle (Steptoe and Edwards, 1978) it soon became evident that this strategy was a very inefficient way of achieving significant pregnancy rates (Jones *et al.*, 1982). Successful in-vitro fertilization (IVF) requires the generation of a number of healthy embryos plus adequate priming and maintenance of a receptive endometrium. Both of these factors are influenced by ovarian stimulation regimes and are responsive to endocrine manipulation. Exogenous gonadotrophins are essential for standard ovulation induction as well as controlled ovarian hyperstimulation in IVF. The first available preparation was human menopausal gonadotrophin (hMG) that contained 75 IU follicle stimulating hormone (FSH) and 75 IU luteinizing hormone (LH), but the majority (> 90%) of the protein content of these preparations consisted of gonadotrophin-unrelated urinary proteins. Thus, during a typical ovarian stimulation cycle with hMG, several milligrams of non-relevant proteins are administered that can result in unwanted side effects including allergic or other hypersensitivity reactions. The next generation of gonadotrophins were the purified urinary FSH or urofollitrophins containing very little LH and non-relevant proteins. This was followed by highly purified urinary FSH containing only traces of LH and finally by the 'pure' preparation of recombinant human FSH. HMG, urinary FSH (uFSH) and recombinant FSH (rFSH) preparations have been used successfully for controlled ovarian hyperstimulation in IVF cycles. Although the fast introduction of rFSH was market driven, its purity, batch-to-batch consistency and availability made it an attractive alternative to the urinary FSH products (Macklon *et al.*, 2006). The purity and batch-to-batch consistency achieved with rFSH had raised hopes of improved outcomes in IVF but disappointingly this has not been proven by trials. Furthermore rFSH is more expensive than uHMG. Hence many units are reverting to the uHMG in IVF cycles. Introducing controlled ovarian hyperstimulation resulted in multi-follicular development and the numbers of retrieved oocytes increased. Premature LH surges during natural or stimulated IVF cycles were responsible for a significant proportion of cycle cancellations. Hence strategies had to be developed to reliably prevent premature LH surges. With the introduction of GnRH agonists in IVF cycles, the incidence of premature LH surges reduced

drastically. This led to a rise in the numbers of retrieved oocytes, number of embryo transfers and evidently in better pregnancy rates (Droesch *et al.*, 1989). For many years GnRH agonists remained the preferred agents for suppression of premature LH surges but more recently GnRH antagonists have been introduced in clinical practice and the diagram below depicts their mechanism of action (Figure 1.3). Various treatment regimes have been evaluated but the one most widely used is the so called 'long protocol' with the use of a GnRH analogue (Figure 1.4).

Figure 1.3 Schematic diagram demonstrating activity of GnRH, GnRH agonist and antagonist on the pituitary gonadotroph cells
A: Effects of GnRH stimulation on release of gonadotrophins

B: Effects of GnRH agonist stimulation on release of gonadotrophins

C: Effects of GnRH antagonist stimulation on release of gonadotrophins

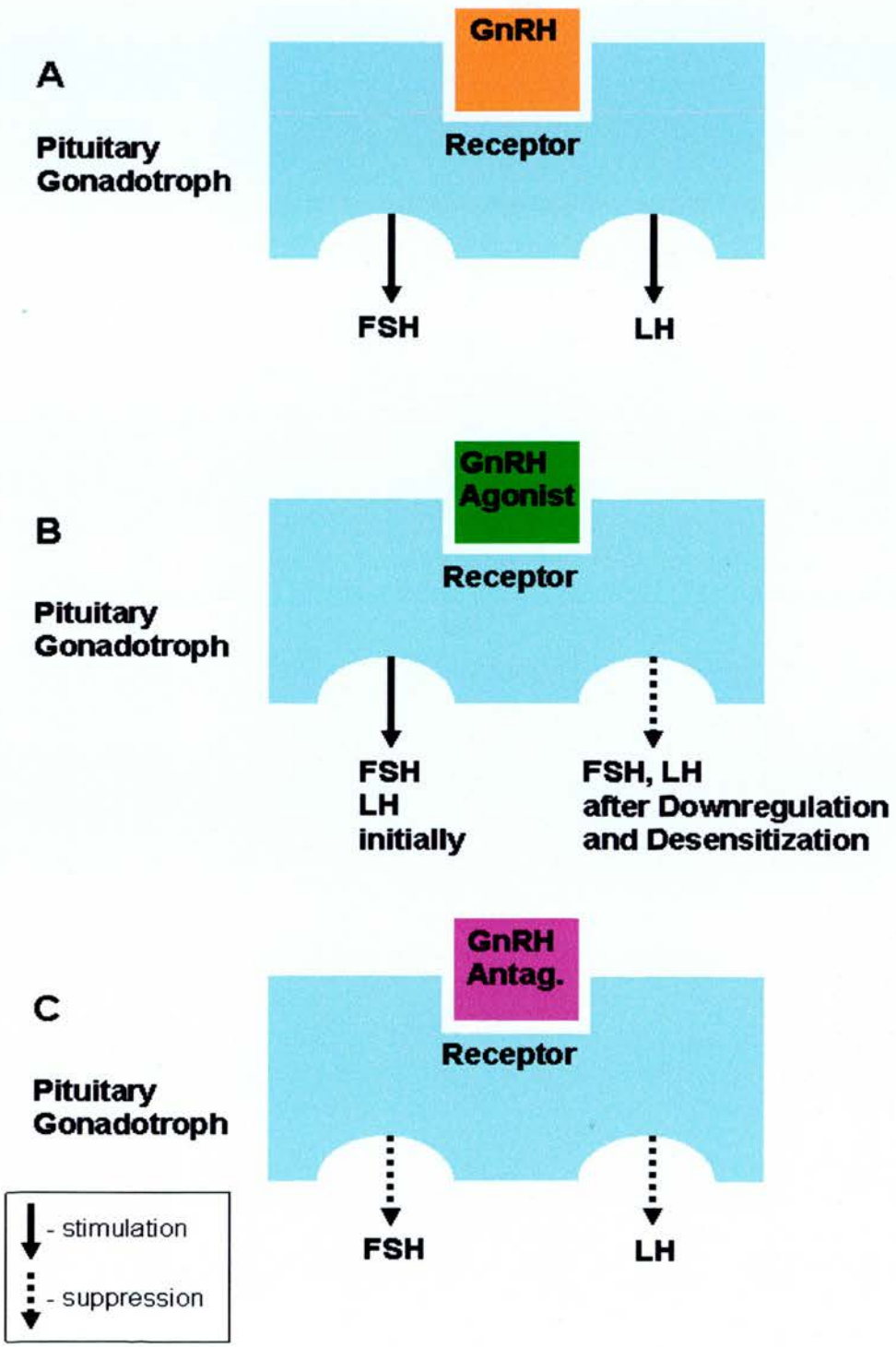
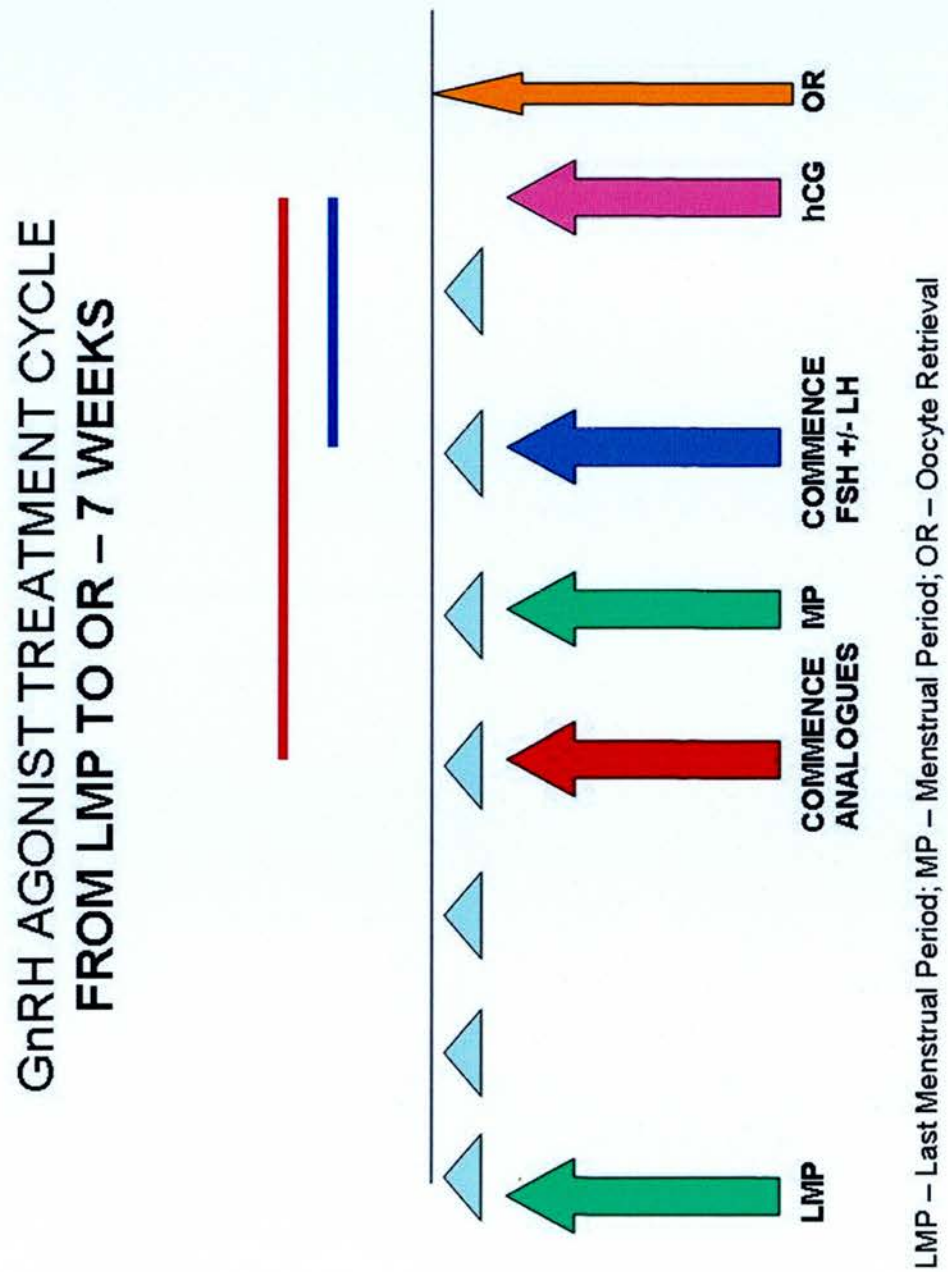


Figure 1.4 Diagram illustrating controlled ovarian hyperstimulation with use of a GnRH agonist – the so called 'long protocol'

Note: Duration of treatment cycle is 7 weeks



This protocol involves starting a GnRH agonist in the mid-luteal stage of a cycle followed by daily administration for approximately 12-14 days. Once pituitary downregulation is achieved, daily injections of urinary or recombinant FSH are commenced. Serial scans are carried out to monitor follicular growth and once at least 3 follicles reach a size of 17 to 18mm, follicular maturation is triggered by a single injection of hCG. Oocytes are retrieved after a period of 34 to 35 hours and IVF/ICSI is carried out. Fertilized embryo(s) or blastocyst(s) are replaced into the endometrial cavity, 2 to 3 days or 5 days after oocyte retrieval respectively. With the use of a GnRH agonist, the average length of a treatment cycle is longer, the amount of gonadotrophins used is higher (Ben-Rafael *et al.*, 1991) and the risk of ovarian hyperstimulation syndrome is greater (Rizk and Smitz, 1992). Furthermore during the phase of pituitary desensitization, there is a period of estrogen deprivation leading, in some women, to menopausal side effects like hot flushes, vaginal dryness and headaches. GnRH antagonist use bypasses some of these disadvantages.

1.2.3.1 Gonadotrophin Releasing Hormone Antagonists (GnRH antag)

As described in section 1.2.1, GnRH is a 10 amino acid peptide secreted in a pulsatile manner by the hypothalamus. GnRH binds to a specific receptor in the pituitary cells to regulate the secretion of FSH and LH. After binding with the receptor, the GnRH-receptor complex elicits several (calcium-dependent) reactions to release FSH and LH (Olivennes *et al.*, 2002).

Recently available GnRH antagonists are GnRH molecules with amino acid modifications at positions 1, 2, 3, 6 and 10. They immediately block the GnRH receptor in a competitive fashion and hence reduce LH and FSH secretion within a period of 8 hours (Olivennes *et al.*, 2002). The inhibition of LH secretion is more pronounced than that of FSH, this being most likely due to the different forms of gonadotrophin regulation, the prolonged FSH half-life or the immunoactive and bioactive forms of FSH (Matikainen *et al.*, 1992).

GnRH antagonists were introduced in clinical practice in the early 1990's (Frydman *et al.*, 1992). They were shown to reliably prevent premature LH surges.

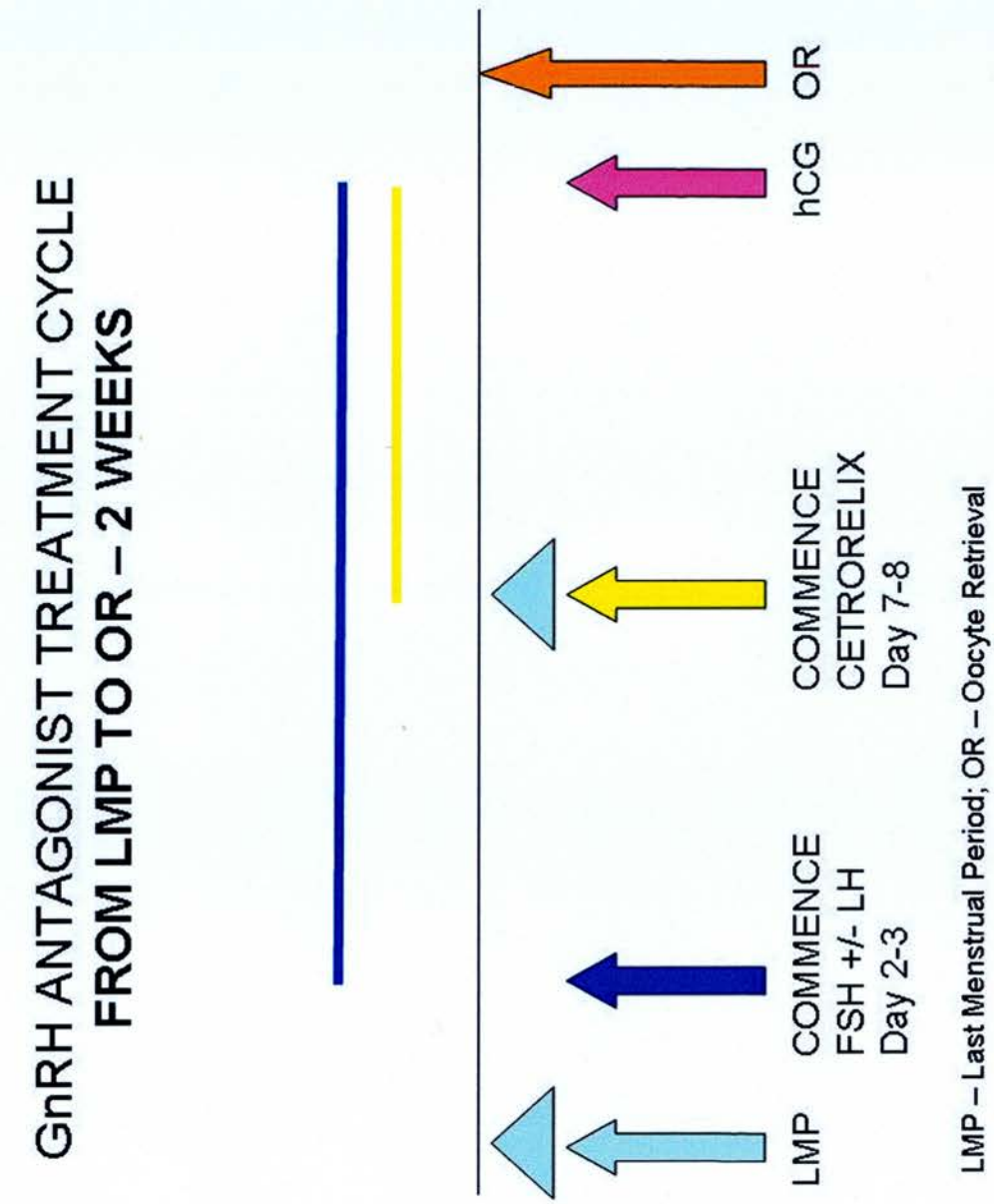
Cetrorelix was the first clinically available GnRH antagonist (Reissmann *et al.*, 2000).

In a GnRH antagonist treatment cycle, urinary or recombinant FSH is started in the early follicular phase. Follicular growth is monitored by serial ultrasound scans. In the mid-follicular phase, once the follicles reach a size of 11 or 12mm, GnRH antagonist is commenced to suppress premature LH surge. Once the follicles reach a size of 17 or 18mm, maturation of the follicles is triggered by a single injection of hCG. Oocytes are retrieved after a period of 34 to 35 hours and IVF/ICSI is carried out. Fertilized embryo(s) or blastocyst(s) are replaced into the endometrial cavity 2 to 3 days or 5 days respectively, after oocyte retrieval (Figure 1.5).

There are several advantages with the use of GnRH antagonists over GnRH agonists. These are namely; shortened duration of treatment cycle, reduction in the amount of gonadotrophins used, avoidance of hypo-estrogenic side effects and a likely reduction in the incidence of ovarian hyperstimulation syndrome (Al-Inany *et al.*, 2006). However, with the use of GnRH antagonists, there are concerns of reduced pregnancy rates (Al-Inany *et al.*, 2006). Hence before GnRH antagonists can replace GnRH agonists in IVF/ICSI treatment cycles, it is vital that we understand the impact of GnRH antagonists on the endometrium in relation embryo implantation. A thorough understanding of the endometrial characteristics under the influence of GnRH antagonists would not only help us understand the processes related to embryo implantation but may also help us devise treatment strategies to reduce the detrimental effects of the hormonal manipulation on the endometrium during IVF/ICSI cycles.

Figure 1.5 Diagram illustrating controlled ovarian hyperstimulation with use of a GnRH antagonist

Note: Duration of treatment cycle is 2 weeks



1.2.4 Steroidogenesis and Steroid Hormones Mechanism of action

The ovary and the adrenals are the classical steroidogenic organs. The steroid hormones and particularly the sex steroids, estrogen and progesterone are released from the ovary. The ovary and the adrenals are the source of the androgens. These hormones in turn act on the endometrium which is the main steroid responsive tissue.

Within the ovary, the theca and the granulosa cells in the developing ovarian follicle are the steroid producing cells. The ovarian steroidogenesis and its control are described in section 1.2.2.

Adrenal glands are the main source of androgens. The adrenal gland is divided into the medulla and cortex. The adrenal cortex is further divided into 3 zones; the outer zona glomerulosa, the zona fasciculate and the inner reticularis. Adrenal androgens are thought to be secreted from the reticularis zone. The adrenal androgens include; testosterone, dihydrotestosterone, androstenedione, dehydroepiandrosterone (DHEA) and DHEA-sulphate (DHEAS). The mechanism of control of androgen secretion is unknown but is thought to be partly related to the Adrenocorticotrophic Hormone (ACTH) secretion (Mckenna and Cunningham, 1991).

The glucorticoids e.g. cortisol and corticosterone are secreted from the inner 2 zones. Cortisol secretion from the adrenals is controlled by ACTH. ACTH is known to be under very sensitive negative feedback control from cortisol, but no such mechanism is thought to exist for adrenal androgens. Therefore it would appear that ACTH plays a role in regulation of adrenal androgens secretion but in conjunction with other factors including prolactin, growth hormone, insulin, sex steroids and a cortical androgen stimulating hormone has been proposed (McKenna *et al.*, 1997).

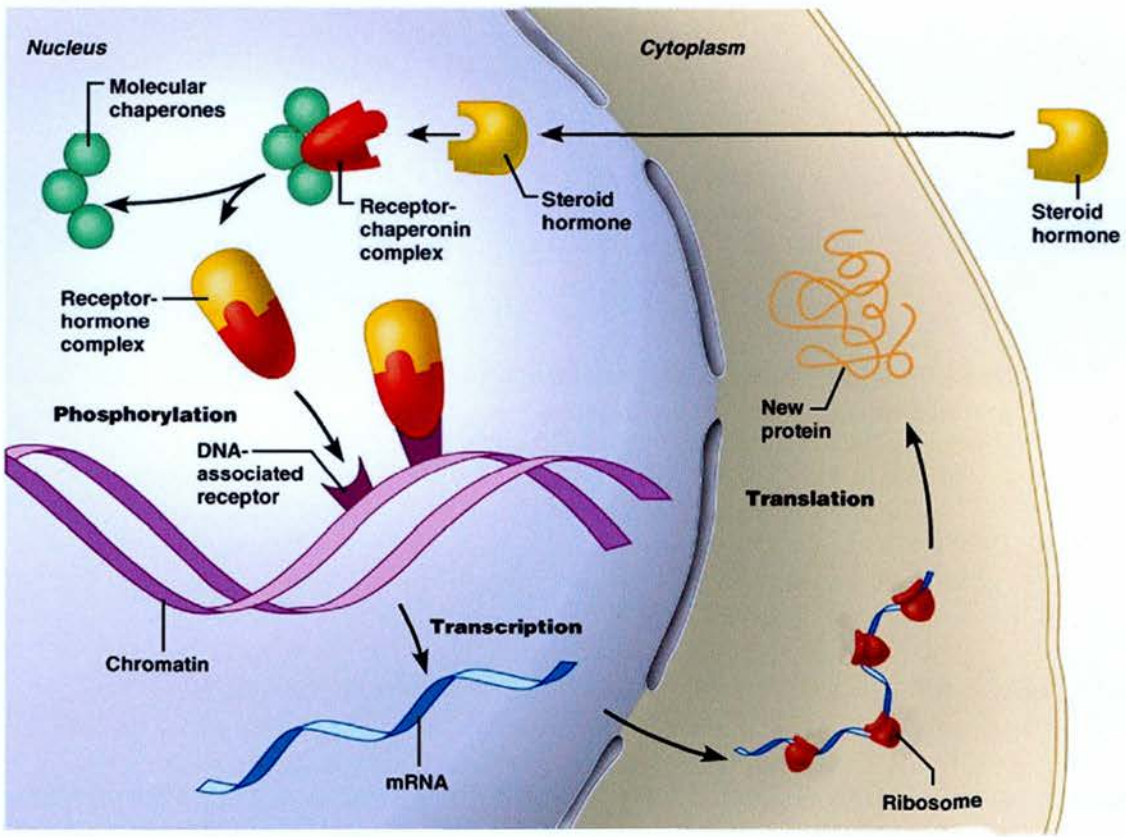
1.2.4.1 Steroid Hormones Mechanism of Action

The steroid ligands classically bind to nuclear receptors, initiating a genomic response (Figure 1.6). The nuclear receptors are principally ligand-inducible transcription factors whose primary function is to mediate the transcriptional response in target cells to hormones such as sex steroids (androgens, estrogens and progestins), adrenal steroids (glucocorticoids, mineralocorticoids) and a variety of

other hormones and metabolic ligands. These protein receptors constitute the Nuclear Receptor Superfamily (Aranda and Pascual, 2001). Members of the Nuclear Receptor Superfamily are divided into 3 subclasses: Type I ('Classical' receptors including AR, PR, ER, GR and Mineralocorticoid receptor), Type II (Thyroid, Retinoic acid and Vitamin D3 receptors) and Orphan receptors with unknown ligands (Aranda and Pascual, 2001). The effects of nuclear receptors on transcription are mediated through recruitment of co-regulators. A subset of receptors binds co-repressor factors and actively represses target gene expression in the absence of ligand. However, upon ligand binding, the receptors undergo a conformational change that allows the recruitment of multiple co-activator complexes. Recruitment of co-activator complexes to the target promoter causes chromatin decompactation and transcriptional activation (Aranda and Pascual, 2001). The characterization of co-repressor and co-activator complexes, in concert with the identification of the specific interaction motifs in the receptors, has demonstrated the existence of a general molecular mechanism by which different receptors elicit their transcriptional responses in target genes.

More recently a further non-genomic action for sex steroids has been recognised. In contrast to genomic responses, non-genomic responses are rapid and their response is not inhibited by agents that inhibit transcription or translation e.g. actinomycin-D or cyclohexamide.

Figure 1.6 Diagram depicting steroid hormone mechanism of action



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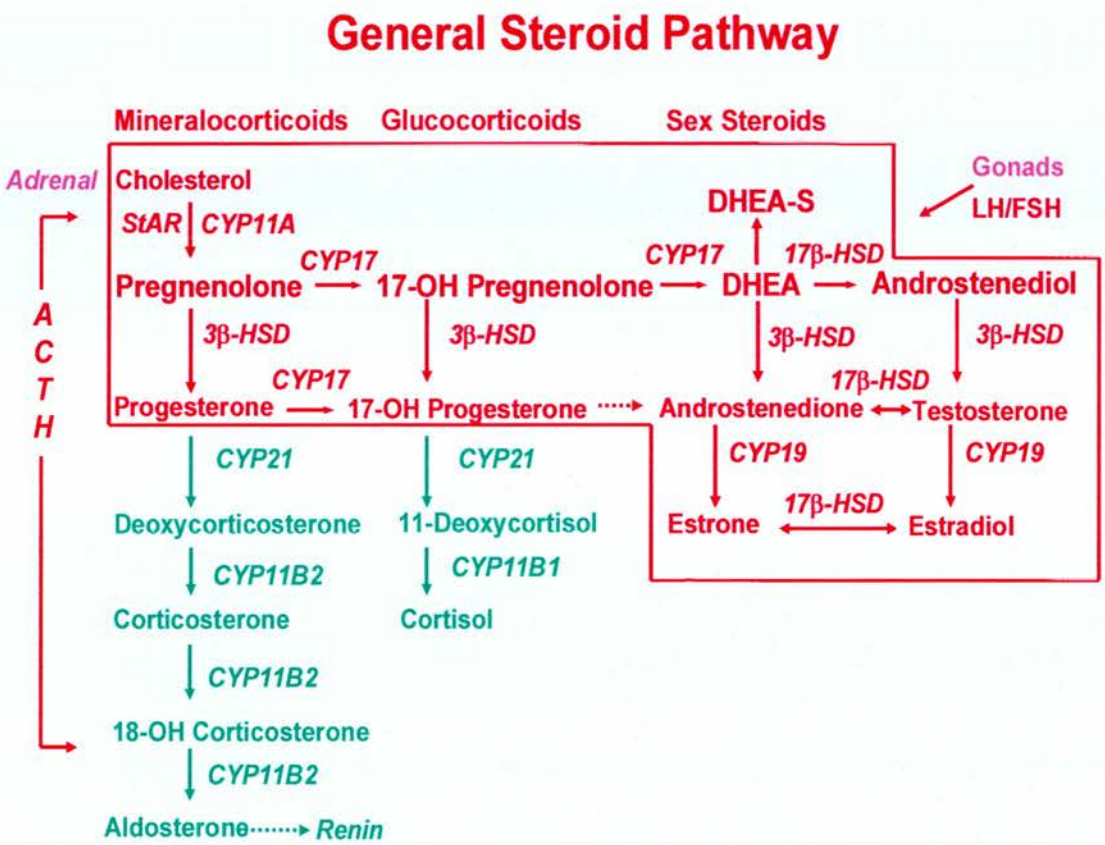
1.3 Intracrinology

Human endocrinology traditionally describes the effects of hormones in relation to their source and their sites of actions. This complex system is controlled by endocrine, paracrine, autocrine and the most recently described intracrine regulation. Classic endocrine systems secrete steroid hormones from the endocrine glands and these are then transported through the systemic circulation to target cells in peripheral tissues. Paracrine systems cause hormone activity on adjacent cells after the release of hormone in the extra cellular space. Autocrine effects refer to actions of the hormone on the same cell as it was released from. Intracrinology refers to the influence of the hormone within the same cell without release into the extra cellular space.

The term 'Intracrinology' was first coined by Labrie and colleagues (Labrie *et al.*, 1988). This important phenomenon conceptualises that the peripheral target tissues such as the endometrium have autonomous control over sex steroid formation and metabolism as per their requirements. Figure 1.7 highlights the intracellular metabolic pathways in the production of sex steroids and the enzymes responsible for their production. In the female, estrogen and progesterone are the main biologically active sex steroids with androgens of presumed secondary importance. The main source of these sex steroids is the ovary, the cyclical production in the ovary being under the control of FSH and LH. After release from the ovary sex steroids are transported to their target site, the endometrium. Whilst some of the intracellular sex steroids in the endometrium could be sourced from the ovary, it is interesting to note that the most abundant sex steroids in the systemic circulation, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulphate (DHEA-S) are both synthesised in large quantities by the adrenal glands under the effect of ACTH. It is these large reservoirs of inactive sex steroids that provide the precursors for conversion into biologically active sex steroids, estrogen and progesterone within the target cell. The target cell responses to steroid hormones are dependent on the target cell concentration of steroid and their binding affinity to the respective receptors. After binding of the ligand, gene transcription is activated thereby leading to the effects of the steroid hormones. Apart from receptor concentration, a key regulatory factor in steroid action is the intracellular

steroid concentration. A significant factor that could affect intracellular steroid concentration is the level and localisation of steroid metabolising enzymes. Hydroxysteroid dehydrogenases (HSDs) regulate ligand access to nuclear receptors in steroid target tissues. This is often achieved by HSDs working in pairs to convert potent steroid hormones to their inactive metabolites and vice versa (Penning *et al.*, 2007). The key enzymes involved in the intracrine metabolism of sex steroids include the isozymes of 17β-hydroxysteroid dehydrogenase (17βHSD), 3β-hydroxysteroid dehydrogenase (3βHSD), 5α-reductase (5αR) and Aromatase. Positional specific HSD pairs have also been proposed to regulate the action of the androgen receptor, the estrogen receptor and the progesterone receptor (Penning, 2003). Whilst the intracrine sex steroid metabolism phenomenon has found effective clinical applications in patients with hormone responsive malignancies like prostate and breast cancers, its role, in another steroid responsive pathology such as endometriosis is only just beginning to receive more attention. It has been proposed that, in endometriotic tissue, a stromal cell defect blocks formation of progesterone-dependent production of factors leading to 17βHSD2 deficiency in turn responsible for a defective estrogen metabolism (Cheng *et al.*, 2007). Matsuzaki and colleagues have found a significantly higher aromatase mRNA expression in epithelial cells than in stromal cells in both eutopic and ectopic endometrium obtained from endometriosis patients. They also observed that in eutopic endometrium from endometriosis patients, 17βHSD2 expression in epithelial cells was significantly increased during the early, middle and late secretory phases compared with the late proliferative phase, whereas no significant cyclical difference was detected in normal endometrium. These findings led them to conclude that local estrogen concentration may be much higher in epithelial cells than in stromal cells in deep endometriotic tissue (Matsuzaki *et al.*, 2006). Studies so far suggest an important role for aromatase inhibitors in the treatment of endometriosis but their exact clinical application in treating endometriosis is yet to be clearly defined (Attar and Bulun, 2006). In the study included in this thesis the endometrial intracrine metabolism of sex steroids in relation to embryo implantation was evaluated.

Figure 1.7 General steroid pathway showing sex steroid synthesis



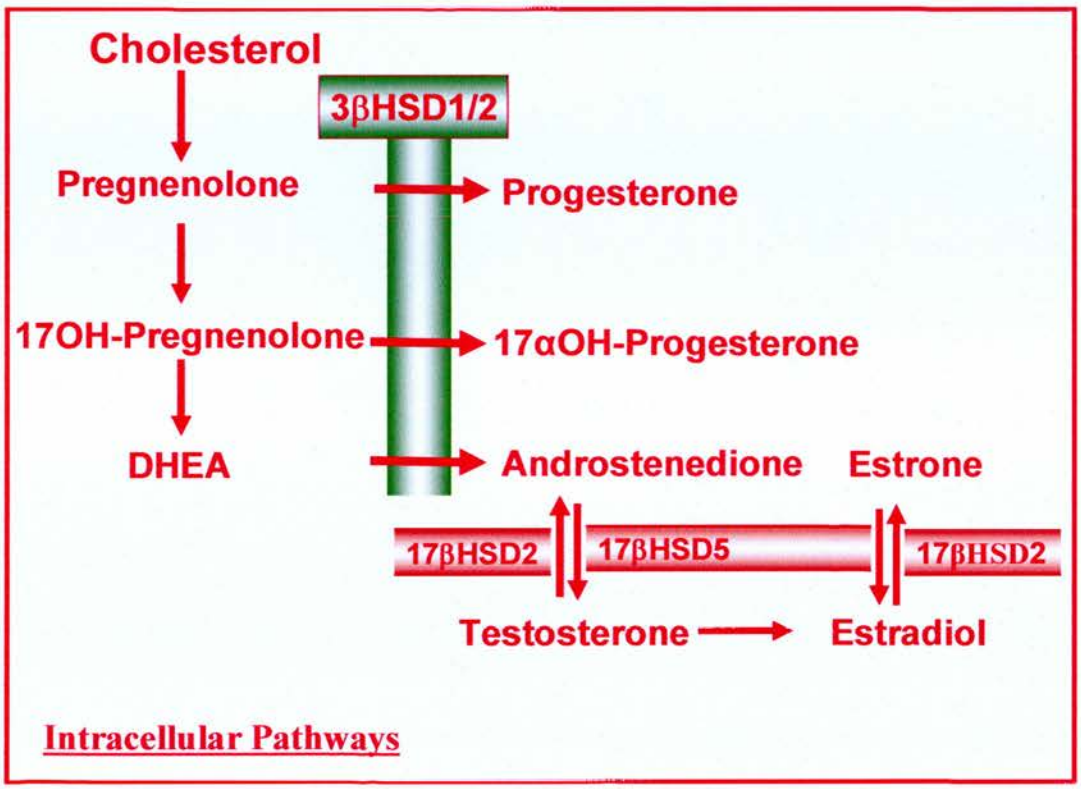
1.3.1 17 beta hydroxysteroid dehydrogenases

The 17β-hydroxysteroid dehydrogenases (17βHSD) are a group of enzymes that catalyze the last steps in the activation and inactivation of estrogens and androgens; the reduction of the 17-keto group to the 17β-hydroxyl on the steroid nucleus, increasing the affinity of the steroids to their cognate receptors (Peltoketo *et al.*, 1996) (Figure 1.8). Type 1 17βHSD, which is abundantly found in the human placenta, was the first isoform to be purified (Karavolas *et al.*, 1970). Since then several isoforms have been identified in various species and currently, there are at least fourteen known isoforms of the 17βHSD enzymes with twelve found in humans (Jansson *et al.*, 2006). In humans they are widely distributed in the body (Labrie *et al.*, 1997). Over and above the classical steroidogenic tissues such as the human placenta, ovary and testis, the 17βHSD enzymes are also found in peripheral intracrine tissues (Martel *et al.*, 1992) including endometrium (Casey *et al.*, 1994) and adipose tissues (Labrie *et al.*, 1997).

The enzymes 17βHSD2 and 17βHSD5 are found in the human endometrium. The enzyme 17βHSD2 is a member of the short chain alcohol dehydrogenase/reductase (SDR) family but 17βHSD5 is a member of the aldoreductase (AKR) protein family (Dufort *et al.*, 1999). The different 17βHSD enzymes, despite catalysing similar reactions often with the same substrates, only share in the region of 20% homology in their gene sequence (Labrie *et al.*, 1997). Estrogens are interchanged by two enzymes, 17βHSD types 1 and 2; type 1 converts Estrone to Estradiol, and type 2 does reverse actions. 17βHSD5 catalyzes the reduction of androstenedione to testosterone and it also converts estrone to estradiol. 17βHSD2 expression was decreased through normal endometrium, hyperplasia and carcinoma accordingly. There was a significant inverse correlation between intratumoral E2 concentration and the level of 17βHSD2 mRNA in endometrial carcinoma. 17βHSD5 expression was significantly increased through normal endometrium, hyperplasia and carcinoma accordingly. These results indicated that 17βHSD types 2 and 5 play an important role in the regulation of in situ estrogen production in endometrial carcinoma (Ito *et al.*, 2006). With the significant effects on intracellular estrogen, progesterone and androgen regulation, a possible role of 17βHSD enzymes in embryo implantation has been explored in animal models (Wu and Matsumoto,

1985; Kreitmann *et al.*, 1980) however till date there are scanty data on human implantation.

Figure 1.8 Biochemical reactions catalysed by the enzymes of the 17β-hydroxysteroid dehydrogenase and 3β-hydroxystroid dehydrogenase enzyme families



1.3.1.1 17βHSD2

The type 2 17βHSD cDNA encodes a predicted protein of 387 amino acids with a molecular mass of 42,782 Da (Wu *et al.*, 1993). The gene is comprised of seven exons which give rise to the types 17βHSD2a and 17βHSD2b proteins. The type 17βHSD2a, but not type 17βHSD2b, has been shown to catalyse the conversion of estradiol to estrone, testosterone into androstenedione and androstenediol into DHEA. It has also shown 20α-hydroxysteroid dehydrogenase activity i.e. the conversion of 20α-dihydroprogesterone to the more active progestin, progesterone (Wu *et al.*, 1993). The function of the 17βHSD2b protein is unknown but it is hypothesized that the type 2b protein might regulate 17βHSD2a activity by forming heterodimers with 17βHSD2a (Labrie *et al.*, 1995). The remaining discussion in this thesis will refer to 17βHSD2a.

The enzyme is a member of the short-chain alcohol dehydrogenase super family. It utilizes NAD as cofactor and preferentially catalyzes the oxidative reaction (Labrie *et al.*, 1997). In the humans it is principally expressed in the placenta (Drolet *et al.*, 2007) but it is also expressed in several other tissues including the endometrium (Maentausta *et al.*, 1991). Gurpide and colleagues first demonstrated the temporal variation in the endometrial expression of 17βHSD2 enzyme. It was shown that the action of progestins up-regulated the activity of enzyme 17βHSD leading to increased conversion of estradiol to estrone in human secretory phase endometrium (Tseng and Gurpide, 1975; Gurpide *et al.*, 1977). More recently it has been demonstrated that this activity is present even in the endometrial epithelial cells and is mediated through a paracrine mechanism from stromal PRs (Yang *et al.*, 2001). In the endometrium, immunostaining of 17HSD appeared in the cytoplasm of surface epithelial and gland cells during the early and midluteal phase with predominant activity in the glandular cells (Maentausta *et al.*, 1991; Scublinsky *et al.*, 1976). During the late luteal phase, it gradually disappeared. No immunostaining was observed in the endometrium during the follicular phase of the menstrual cycle (Maentausta *et al.*, 1991). The highest levels of 17βHSD2 mRNA were found in endometrial tissues obtained during the mid- to late secretory phase of the ovarian cycle (i.e., during the time of high plasma levels of progesterone). 17βHSD2 mRNA levels were much greater in glandular epithelium

than in the stromal cells isolated from secretory phase endometrium (Casey *et al.*, 1994).

The precise function of 17βHSD2 in human endometrium is unknown. It is hypothesized that the effects during the luteal phase are not only aimed at the down-regulation of estrogen action but also to up-regulate progesterone action (Tseng and Gurpide, 1975; Mustonen *et al.*, 1998). Furthermore through its effects on the androgen regulation it is likely to have a significant impact on the intracellular steroid milieu predominantly in the mid-luteal phase of the menstrual cycle. Hence it is vital, particularly in relation to embryo implantation, to gain a clear understanding of the role of 17βHSD2 in human endometrium.

1.3.1.2 17βHSD5

In contrast to the other 17-hydroxysteroid dehydrogenases, 17βHSD5 belongs to the aldo-keto reductase family. It was originally cloned from the human liver but was then demonstrated in multiple organs like brain, kidney, testis and placenta. During the earlier stages of its discovery it was labelled as 3α-hydroxysteroid dehydrogenase type 2 (Khanna *et al.*, 1995). However Dufort and colleagues established that human 3α-HSD2 was not the predominant activity and a higher percentage conversion of androstenedione to testosterone and progesterone to 20α-hydroxyprogesterone occurred when a stably expressed enzyme was studied. It was felt from this and prior studies that homogenization protocols had destroyed much of the 17β-HSD activity of the enzyme, suggesting that it was highly labile (Dufort *et al.*, 1999). Therefore it is now recognised as 17βHSD5.

The 17βHSD5 activity of the enzyme converts androstenedione to testosterone and the 20α-hydrogenase activity will inactivate progesterone to 20α-hydroxyprogesterone (Penning *et al.*, 2001; Dufort *et al.*, 1999). The double activity of type 5 17βHSD in the female reproductive tissues is probably necessary for the control of optimal level of progesterone and sex steroids (Luu-The *et al.*, 2001). Human 17βHSD5 possesses a high 20α-HSD activity that inactivates progesterone, whereas the monkey and mouse enzymes do not have such high 20α-HSD activity (Luu-The *et al.*, 2001) and hence some of the activities of the enzyme appear to be species specific.

In the female 17βHSD5 has been localised in the ovary, breast and the uterus (Pelletier *et al.*, 1999). In the uterus, 17βHSD5 was localised to the endometrium, the myometrium being completely unlabeled. Surface epithelial cells and glandular cells were strongly reactive. The endometrial stroma was devoid of labelling (Pelletier *et al.*, 1999). The endometrial AR is predominantly stromal (Burton *et al.*, 2003) and hence it is possible that the 17βHSD5 androgen production produces a paracrine effect. Alternatively, its role may relate to its 20α-HSD activity, inactivating progesterone. As has been suggested, the activity of type 5 17βHSD in the female reproductive tissues is probably necessary to control optimal levels of progesterone and sex steroids, changes in this enzyme could potentially alter the mid-luteal availability of ligands thereby affecting implantation. Hence further clarification of 17βHSD5 activity in the mid-luteal stage is vital.

1.3.2 3 beta Hydroxysteroid Dehydrogenases

The enzyme 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3βHSD) is involved in the biosynthesis of all classes of active steroids namely progesterone, glucocorticoids, mineralocorticoids, androgens and estrogens (Mason *et al.*, 1997; Labrie *et al.*, 1992) (Figure 1.8). Two isozymes are present in the humans and both are encoded by two distinct genes. The 3βHSD1 gene encodes an enzyme of 372 amino acids predominantly expressed in the placenta and peripheral tissues, such as the skin, mammary gland, prostate, endometrium and several other normal and tumour tissues (Thomas *et al.*, 2001; Simard *et al.*, 2005). The type 2 gene, which encodes a protein of 371 amino acids, shares 93.5% identity with the type 1 enzyme and is predominantly found in the adrenals and the gonads (Thomas *et al.*, 2001; Simard *et al.*, 2005). The human 3βHSD1 cDNA was first isolated and characterized by Luu-The and colleagues (Luu-The *et al.*, 1989). The second human 3βHSD isoenzyme, chronologically designated as type 2, was isolated from a human adrenal cDNA library (Rheaume *et al.*, 1991).

3βHSD activity has been described in the human endometrium (Tang *et al.*, 1993). Its role in human embryo implantation has been proposed but there are few data in relation to human pregnancy (Rhee *et al.*, 2003). Immunohistochemistry has shown that in the secretory phase 3βHSD is moderately expressed in the glandular

epithelium of the endometrium with very little expression in the proliferative phase (Rhee *et al.*, 2003). In the human endometrium 3βHSD is thought to catalyze the reaction that converts pregnenolone to progesterone (Figure 1.8) and it has been postulated that the de novo synthesis of progesterone in the endometrium might be crucial for implantation and maintenance of pregnancy. The type of 3βHSD found in the endometrium is type 1, the same type that is predominantly localised in the placenta (Rhee *et al.*, 2003).

1.4 The human uterus and endometrium

The uterus is a dynamic endocrine organ derived from the mullerian duct. Grossly it is divided into 3 anatomical parts: the body of the uterus, the isthmic portion and the cervix. It is a fibro-muscular organ that is essential for reproduction. It is composed of an outer layer made up of smooth muscle and the inner cellular layer, the endometrium. The smooth muscle cells of the uterus undergo hypertrophic and hyperplastic changes under the effect of sex hormones.

At various stages in life, in response to sex hormones, the uterus undergoes periodic changes. Pre-pubertal, the body of the uterus is in equal proportion to the size of the cervix. With the onset of puberty and with the cyclical changes in the female hormones, the body of the uterus enlarges to double the size in relation to the cervix. Ultrasound based studies have shown that the uterine size and the ovarian volume increase before the onset of clinical puberty and these processes continue well into the second decade (Holm *et al.*, 1995). Pregnancy leads to considerable changes in the uterus. The smooth muscle cells of the myometrium undergo significant hypertrophy, hyperplasia and remodelling under the influence of ovarian and placental steroids ensuring the implantation of the embryo and then the growth of the fetus. With the depletion of the sex steroids during menopause, the smooth muscle cells undergo atrophy leading to shrinkage in the size of the uterus.

The endometrium is the most dynamic hormone responsive element of the uterus. It lines the body of the uterus and it must cyclically undergo regeneration and remodelling in each menstrual cycle in preparation for possible embryo implantation. Histologically, human endometrium can be identified as 3 layers: the

superficial zona compacta, the intermediate zona spongiosa and an inner basal layer. The inner basal layer is in contact with the myometrium. It is not shed during menstruation and from this layer starts the regeneration of the endometrium during each menstrual cycle. The superficial layers i.e. zona compacta and spongiosa, are referred to as the functional layers of the endometrium. The cellular compartments that can be identified in the endometrium are the superficial epithelium, the stroma, the glands, the vascular endothelium and the perivascular cells. The ovarian steroids act on the cells of these compartments to effect the cyclical changes throughout the menstrual cycle.

1.4.1 Endometrial morphology during the menstrual cycle

The first half of the menstrual cycle is referred to as the proliferative phase and this is predominantly estrogen driven. After ovulation the progesterone driven phase starts which leads to secretory differentiation i.e. the secretory phase, of the endometrium. In the absence of conception, following luteolysis, shedding of the functional layers of the endometrium occurs against a background of falling levels of progesterone and estrogen as a consequence of luteal regression. This is referred to as the menstrual phase.

Noyes et al., in their seminal paper, described the endometrial morphology at each stage of the menstrual cycle. They divided the proliferative and secretory phases further into early, mid and late categories and produced a guide for dating the endometrium. Even today this system forms the basis of dating the endometrium (Noyes *et al.*, 1950). Relevant details of the characteristics of endometrium at each stage of the cycle are mentioned below (Noyes *et al.*, 1950; Speroff and Fritz, 2005; Buckley and Fox, 1989).

Proliferative Phase

This stage is characterised by regenerating columnar surface epithelium. Initially the glands are narrow and tubular. Then mitoses become prominent, pseudo stratification is observed and by linking one gland segment with another a continuous epithelial lining facing the endometrial cavity is formed (Speroff and Fritz, 2005). The spiral vessels course through the stroma to reach a point

immediately beneath the epithelial binding membrane where they form a loose capillary network. All the tissue components (glands, stromal cells and endothelial cells) demonstrate proliferation, which peaks on days 8-10 of the cycle, corresponding to peak estradiol levels in the circulation and maximal estrogen receptor concentration in the endometrium (Bergeron *et al.*, 1988).

Secretory Phase

The appearance of subnuclear glycogen-rich vacuoles in the glandular epithelium of at least 50% of cells is a prominent feature of early secretory phase endometrium (Buckley and Fox, 1989). Epithelial proliferation ceases after ovulation and this inhibitory effect is believed to be under the influence of progesterone. Mitoses cease to be seen. Glands become more tortuous and intra-luminal secretions reach a peak. Stromal oedema is marked. The spiral arterioles become much more prominent with intensified coiling. These changes are the first signs of decidualization. In the absence of conception, in late secretory phase, there is marked physiological leucocyte infiltration. There is a stromal regression and loss of endometrial height. The gland secretion diminishes to a variable extent and the involution of gland epithelium occurs. The epithelium is thrown into tufts giving rise to the characteristic saw-toothed effect.

Menstrual Phase

The withdrawal of estrogen and progesterone initiates important endometrial events which culminate in menstruation. The most prominent effects of the hormone withdrawal are evident on the spiral arterioles. With progressively prolonged and profound arteriolar spasms, endometrial ischaemia results followed by breakdown and shedding of the superficial layer, upper two thirds, of the endometrium. The endometrium at this stage exhibits focal areas of sub-epithelial necrosis with subsequent glandular collapse and necrosis. The basal layer of the endometrium remains and repair recommences from this layer.

1.4.2 Endometrial morphology during mid-secretory phase (window of implantation)

During the secretory phase, the endometrium is predominantly influenced by the progesterone produced by the corpus luteum in the ovary. The changes in the endometrium are tightly regulated and it is generally accepted that optimum conditions need to be in place during a definite period commonly referred to as the 'window of implantation' for successful implantation of the embryo. This putative 'window' is thought to extend from days LH+6 to LH+10.

The most profound and documented effects are seen in the endometrial glands but all cell types undergo significant changes. On day LH+5, mitoses have ceased in the endometrial glandular cells but are still occasionally evident in the stroma.

Endometrial glands during the window of implantation (Glasser et al., 2002):

The secretory products in the glands increase and the secretory apparatus increases to a maximum by LH+6. The glands become more tortuous. On day LH+7 the gland cells contain little secretory material and have acquired a low columnar to cuboidal appearance and this contributes to the saw tooth appearance of the glands.

Endometrial blood vessels during the window of implantation (Glasser et al., 2002):

At this stage the spiral vessels are prominent and densely coiled. Progesterone is responsible for an increase in the dilatation of endometrial subepithelial blood vessels. The significance of this vascular dilatation is uncertain. Morphometric analysis has shown that between LH+6 and LH+12, the proportion of endometrium made up of blood vessels remains constant (Rogers *et al.*, 1993). Electron microscopy of endothelial cell profiles in small blood vessels demonstrated that their nuclear diameter did not change but the proportion of rough endoplasmic reticulum, secretory apparatus and mitochondria increased between LH+6 and LH+10.

Endometrial stroma during the window of implantation (Glasser et al., 2002; Neill, 2006):

The endometrial stromal cells are now thought to arise from the primitive uterine mesenchymal stem cells. Around the time of implantation, the stromal cells become large and polyhedral. Beginning on day LH+6 and thereafter in the cycle,

changes in the stroma become prominent. Stromal changes include stromal edema, increased capillary permeability, stromal mitoses, endothelial cell proliferation and coiling of the spiral arterioles. Maximal capillary permeability and stromal edema occur on day LH+10 and are prerequisites for predecidual transformation of endometrial stromal fibroblasts.

Endometrial luminal epithelium during the window of implantation (Neill, 2006):

The predominant feature on the endometrial epithelium is the appearance and then disappearance of pinopodes. These structures, visualised through electron microscopy, are important for embryonic attachment. During the window of implantation, they persist for 1-2 days and their presence positively correlates with implantation sites. Continuing pregnancy rates are high for embryos that implant between Post Ovulation Days 8-10 (84%) compared to 18% when implantation occurs 11 days or more after ovulation (Wilcox *et al.*, 1999).

1.4.3 Endometrial morphology of the postmenopausal endometrium

There are limited data on the histology of the postmenopausal endometrium. A likely cause may be the difficulty in obtaining sufficient tissue for analysis. However Archer et al, in their study that analysed 801 endometrial biopsies from asymptomatic perimenopausal and postmenopausal women found that the endometrium was atrophic in 373 (46.9%), proliferative in 133 (16.7%), secretory in 54 (6.8%), and hyperplastic in 41 (5.2%). Insufficient tissue for diagnosis was obtained in 195 (24.5%) (Archer *et al.*, 1991). Another larger study that included endometrial biopsies from 2964 perimenopausal and postmenopausal women prior to starting HRT, showed 68.7% were atrophic, 23.5% were proliferative, 0.5% were secretory, 0.6% were hyperplastic, 0.07% were adenocarcinoma, and 6.6% were insufficient for classification (Korhonen *et al.*, 1997). Noci et al, in a small study, observed a discrepancy between the morphology and the immunohistochemical analysis of postmenopausal endometrium. Whilst the morphological pictures suggested an involution of this tissue according to the increase in collagen fibres, the decrease in vascular distribution and the frequent atrophic patterns, on the other hand, data from steroid receptors and the cell proliferation index suggested that post-menopausal endometrium is an active

structure. So they conclude that the endometria from normal post-menopausal women appear to be in a more quiescent state than in a really atrophic condition (Noci *et al.*, 1996).

Postmenopausal endometrium, especially in the absence of abnormality, is a challenging area for research, not least because of a significant proportion of endometrial biopsies not yielding sufficient tissue for analysis.

1.5 Endometrial Expression of Steroid Receptors, Proliferation Markers and Decidualisation Markers

The endometrial proliferation, secretory transformation, shedding and repair are all processes under the control of steroid hormones. The steroid hormones act through their cognate receptors. In an attempt to unravel the specific functions of each steroid hormone, research has been directed at elucidating the temporal and spatial expression of the receptors of these hormones in the endometrium. The endometrial expression of estrogen and progesterone receptors have been best characterised. Expression patterns of AR and GR have only been studied more recently. Furthermore with increasing interest in the field of endometrial biology, subtypes and variants of the well characterised ER and PR have been identified. Hence further studies will be needed to clarify their distribution and ultimately their effects on endometrial physiology and pathology.

The endometrium is a very dynamic tissue undergoing cyclical shedding and repair. This involves processes of cellular proliferation and apoptosis. Markers to study these processes are increasingly being recognised but their exact roles in various endometrial functions are yet to be understood. Current understanding regarding the endometrial function of 3 such markers; Ki-67, phosphorylated Histone-3 (PH3) and Insulin like Growth Factor Binding Protein -1 (IGFBP-1) is presented below.

1.5.1 Estrogen and Progesterone Receptor Expression and Regulation

Since the ER and PR expression is interlinked, these are reviewed together in this section. With the development of specific monoclonal antibodies to ER and PR and with improved antigen retrieval techniques, progress has been made in

immunolocalisation of these receptors. Immunohistochemistry has been extensively used for studies to determine the receptor distribution in the endometrium. The ER and PR are immunolocalised to the nuclear compartments of the cells. Their spatial localisation is broadly described in relation to the superficial and basal layers and further categorised into the epithelial, stromal, glandular, vascular endothelial and perivascular regions.

There are several isoforms of human PR (Taylor *et al.*, 2006). In relation to the human endometrium, the two classical isoforms of PR are PR-A and PR-B. These are known to arise from the same gene (Graham and Clarke, 1997). The A isoform which is 769 amino acids (aa) in length is a truncated version of the B isoform that is 933 aa in length, the truncation of 164 aa being from the N terminal end of the protein (Sartorius *et al.*, 1994). With the development of two anti-progesterone receptor antibodies, a specific polyclonal raised against PR-B and the other a commercially available monoclonal raised against PR subtype A+B, it has been possible to refine the cellular localization of PR subtype A and B in the normal endometrium throughout the menstrual cycle. Since PR-A is a shorter splice variant it is not possible to raise a specific antibody to this isoform alone. Hence immunohistochemical analysis of PR-A may be further determined by subtractive inference (Wang *et al.*, 1998).

In relation to the ER, two distinct isoforms, ERα and ERβ have been identified. Whilst the two isoforms of PR arise from the same gene, the 2 identified isoforms of ER, ERα and ERβ, arise from two different genes (Koike *et al.*, 1987; Kuiper *et al.*, 1996, Enmark *et al.*, 1997). The recently discovered ERβ was initially cloned in the rat but has since been cloned in the humans with the classical ER now designated ERα (Mosselman *et al.*, 1996).

By comparing monoclonal antibody immunolocalisation with ligand binding studies, Lessey et al described the temporal expression of ER and PR (Lessey *et al.*, 1988). These data showed an increase in the concentration of ER throughout the proliferative phase with a peak in the early secretory phase. Thereafter the ER concentrations decline. The expression of PR, and therefore sensitivity to progestins, is under the control of estrogen, which increases, and progesterone, which decreases PR expression in most target tissues (Graham and Clarke, 1997).

Concentrations of PR increase in a parallel fashion, albeit somewhat delayed, with a decline in the secretory phase of the cycle. The nuclear localisation of ER and PR in the endometrial compartments has been demonstrated by immunohistochemical studies. In the stromal and glandular compartments, ER expression was maximal in the late proliferative phase. However, in the secretory phase, ER expression declined in the stromal and glandular compartments. It is also possible to localise ERα and ERβ using monoclonal antibodies. ERα and ERβ are both localised in the nuclei of the glandular compartment and stromal cells of functional and basal endometrium in the proliferative phase. The expression of ERα declines in the glands and stroma of the functional layer while expression of ERβ declines in the glands, but not the stroma, during the secretory phase. Levels of expression of ERα and ERβ in all cellular compartments remain unchanged in the basal layer (Critchley *et al.*, 2001). Marked PR expression is observed in the nuclei of glands and stroma during the proliferative phase. In the secretory phase this immunoreactivity declines in the glandular cells with a modest expression in stromal cells (Lessey *et al.*, 1988; Snijders *et al.*, 1992; Critchley *et al.*, 1993). In the glands, PRA and PRB were expressed before subnuclear vacuole formation and glycogenolysis, implicating both isoforms in this process, whereas persistence of PRB during the midsecretory phase suggested its significance in glandular secretion. In the stroma, the predominance of PRA throughout the cycle implicates this isoform in post-ovulatory progesterone-mediated events. These results support the view that PRA and PRB mediate distinct pathways of progesterone action in the glandular epithelium and stroma of the human uterus throughout the menstrual cycle (Mote *et al.*, 1999). Using subtractive inference it was determined that the isoform of PR that mainly persists in the secretory phase stroma is PRA (Wang *et al.*, 1998). A study by Brosens and colleagues provides further support to this observation but they also demonstrated that whilst PRA was the predominant isoform in differentiating endometrial stromal cells, its abundance decreased markedly during the course of the decidualization response (Brosens *et al.*, 1999).

In relation to endometrial vascular function, ER and PR expression show some interesting features. Both PRA and PRB are present in a perivascular location in the secretory phase, but not in the proliferative phase. The presence of PRA and

PRB in the secretory phase makes it likely that progesterone action either directly or indirectly influences growth, development and function of the spiral arterioles in this phase of the menstrual cycle (Koji *et al.*, 1994; Wang *et al.*, 1998). It appears that the estrogen mediated regulation of the endometrial vasculature and angiogenesis may be mediated via binding to ERβ. This is because only ERβ, and not ERα, is present in the nuclei of the endothelial cells of most blood vessels including spiral arterioles, capillaries and veins. The intensity of immunoreactivity of ERβ in the endothelial cells shows little change throughout the proliferative and secretory phases (Critchley *et al.*, 2001).

1.5.1.1 Estrogen receptor regulation

ER and PR regulation is closely interlinked. Estradiol stimulates ER expression and ER levels are highest in the proliferative phase (Lessey *et al.*, 1988). ER expression decreases after ovulation, reflecting the suppressive effects of progesterone on ER expression. Two forms of ER are now recognised, ERα and ERβ. The functional significance of ER is gradually being unravelled with the generation of ER knock out (ERKO) mouse models. At the time when the first ER knock out mouse was generated (Lubahn *et al.*, 1993), the only ER isoform known was ERα. The initial studies on αERKO mice showed interesting features. Both male and female animals survived to adulthood with normal gross external phenotypes. Females were infertile; males had a decreased fertility. Females had hypoplastic uteri and hyperemic ovaries with no detectable corpora lutea (Lubahn *et al.*, 1993). ERβ knock out mice were generated 5 years later by which stage the second ER isoform had been recognised. In the initial studies involving βERKO mice it was observed that ERβ was essential for normal ovulation efficiency but not essential for female or male sexual differentiation, fertility or lactation (Krege *et al.*, 1998).

In the endometrium, ERβ expression is lower than ERα and in the ERα null mouse, ERβ does not compensate for ERα (Couse and Korach, 1999). This suggests that ERα is the predominant ER in the endometrium. However only ERβ, but not ERα, is present in the endometrial vascular endothelium (Critchley *et al.*, 2001) and hence the vascular effects of estrogens are directly mediated via this receptor isoform.

Estradiol action on the endometrium is direct, through its cognate receptor and indirect, by induction of growth factors in either target cells or by inducing growth factors as paracrine modulators. In relation to cell-cycle regulation, estradiol complexed with ER leads to up-regulation of cell cycle proteins. In the proliferative phase, an increase in ERα expression is correlated with cyclins and cyclin-dependant kinases. Furthermore, the transcription factors c-fos and c-jun, mediators of estradiol action, are highly expressed in the proliferative phase (Neill, 2006).

1.5.1.2 Progesterone receptor regulation

The PR is controlled by both estrogen and its own ligand progesterone. In the proliferative phase, which is predominantly estrogen dominated, PR is upregulated secondary to the estradiol up regulation of PR mRNA. This effect occurs as a result of an estrogen response element that results in increased PR protein synthesis (Clarke and Sutherland, 1990). In the secretory phase, which is progesterone dominated, progesterone itself initiates down regulation at both the transcriptional, via the same estrogen response element (Savouret *et al.*, 1991), and post transcriptional levels (Chauchereau *et al.*, 1992).

Just as in ER, two isoforms of PR are currently recognised, PRA and PRB. While progesterone action on target genes is conferred primarily by PRB homodimers, truncated PRA acts as a repressor of PRB function (Neill, 2006).

To better understand the functional significance of PR, PR knock out (PRKO) mice were generated. Initial studies on these mice demonstrated that male and female embryos homozygous for the PR mutation developed normally until adulthood. However, the adult female PR mutant displayed significant defects in all reproductive tissues. These include an inability to ovulate, uterine hyperplasia and inflammation, severely limited mammary gland development and an inability to exhibit sexual behaviour. Collectively, these results provided direct support for progesterone's role as a pleiotropic coordinator of diverse reproductive events that together ensure species survival (Lydon *et al.*, 1995). Estradiol treatment of the PRAKO mouse results in endometrial epithelial hyperplasia and progesterone treatment had no subsequent effect, suggesting that PRA mediates progesterone inhibition of estradiol stimulated endometrial epithelial growth. Ablation of PRB

protein (PRBKO mice) does not affect biological responses of the ovary or uterus to progesterone but results in reduced pregnancy-associated mammary gland morphogenesis (Mulac-Jericevic and Conneely, 2004). The use of mifepristone, a progesterone receptor antagonist has further elucidated progesterone regulated events in the endometrium. Mifepristone given early in the secretory phase in humans prevents progesterone-induced down-regulation of PR and ER (Cameron *et al.*, 1996; Maentausta *et al.*, 1993).

1.5.2 Androgen Receptor Expression and Regulation

Two isoforms of AR have been proposed. An AR-A isoform (ARA) with an apparent molecular mass of 87 kDa, which is an NH_2-terminally truncated form of a major AR-B isoform (ARB), was reported (Wilson and McPhaul, 1996; Gao and McPhaul, 1998). Although the study suggested that the use of initiation methionine at amino acid 188 would yield the ARA isoform, the possibility that the ARA isoform might be a proteolyzed product of AR could not be ruled out. As no follow-up studies were reported about the N-terminally truncated isoform of AR, the concept of AR isoforms is not widely accepted (Lee and Chang, 2003). This is in contrast to other steroid receptors, such as PR and ER.

In comparison to ER and PR, in the human endometrium, AR has been less extensively studied. This may be partly related to the fact that there are a variety of antibodies and immunohistochemical techniques employed to study AR in the human endometrium. Initially, the presence of an androgen receptor in the human endometrium was shown with ligand binding studies (Muechler, 1987). However subsequent immunolocalisation studies using the mouse monoclonal antibody (F39.4) localised AR in a variety of human tissues, but no receptors were identified in the uterus (Ruizeveld de Winter *et al.*, 1991). Horie et al, using the rat anti-human monoclonal antibody, found the AR to be present in endometrial glands and stroma throughout the menstrual cycle (Horie *et al.*, 1992). The immunoreactivity was mainly localised to the functional layer with less in the basal layer (Horie *et al.*, 1992). Other studies have found immunoreactivity in the endometrial stroma and glands (Kimura *et al.*, 1993; Takeda *et al.*, 1990) but the level of glandular expression has been variable with Takeda et al reporting some expression and

Chadha et al reporting no expression in the endometrial glands (Takeda *et al.*, 1990; Chadha *et al.*, 1994). Mertens et al localised the receptor in the stromal cells with the most profound immunoreactivity in the basal layer and only minimal immunoreactivity in the glandular epithelium. In this study and in the one by Slayden and colleagues, AR immunoreactivity was not observed in the late secretory phase indicating there may be temporal variation in expression within the menstrual cycle (Mertens *et al.*, 1996; Slayden *et al.*, 2001b).

1.5.2.1 Androgen receptor regulation

The functional significance of the androgen receptor pathway in the male reproductive system is well studied. However, until the recent past in the absence of a proper model system, the AR pathway in the female is unclear. Recently, AR knockout (ARKO) mice have been successfully generated (Yeh *et al.*, 2002). The study demonstrated that the average number of pups per litter in homologous and heterozygous ARKO female mice was lower than in wild type female mice, suggesting potential defects in female fertility and/or ovulation (Yeh *et al.*, 2002). It has also been reported that the ovaries from sexually mature ARKO female mice exhibited a marked reduction in the number of corpora lutea. During the periovulatory period, in the ARKO female mice, an intensive granulosa apoptosis event occurs in the preovulatory follicles, concurrent with the down-regulation of p21 and progesterone receptor expression. In addition to insufficient progesterone production, the diminished endometrial growth in uteri in response to exogenous gonadotropins indicates that ARKO females exhibit a luteal phase defect (Hu *et al.*, 2004).

Mertens et al observed AR downregulation in the late secretory phase (Mertens *et al.*, 1996; Mertens *et al.*, 2001). Since this downregulation was observed in a progesterone dominated phase, it may be suggested that the downregulation is secondary to a progesterone mediated mechanism. Fujimoto et al studied women given an intramuscular injection of estradiol dipropionate (ED) with or without testosterone cypionate (TC) 1 week prior to hysterectomy. ED alone was responsible for a significant increase in AR mRNA and testosterone binding sites but when administered with TC the testosterone binding sites were significantly

reduced and the AR mRNA level showed a non significant increase suggesting that while TC significantly reduced testosterone binding sites it had no significant effect on AR mRNA levels (Fujimoto *et al.*, 1995). Iwai et al studied endometrial stromal cell cultures and found that progesterone and testosterone independently were able to decrease ER mRNA, PR mRNA and AR mRNA in a dose dependent manner (Iwai *et al.*, 1995). There are scanty data in the field of AR regulation and indeed further work is required in this field.

1.5.3 Glucocorticoid Receptor Expression

Glucorticoids exert their effects on human tissues through the GR. In humans, two receptor isoforms, hGRα and hGRβ, have been identified. These originate from the same gene by alternative splicing (Encio and Detera-Wadleigh, 1991). The two receptor isoforms are identical between amino acid 1–727 and then diverge. hGRα contains 50 specific C-terminal amino acid residues that form two helical structures that play an important role in ligand binding, whereas the C terminus of hGRβ contains 15 specific nonhomologous amino acids which are largely disordered (Yudt *et al.*, 2003). Until now, hGRβ has been only found in the humans and hence no animal models were available for further studies. Recently, hGRβ has been reported in other species potentially providing animal models for further studies on GR mediated effects (Schaaf *et al.*, 2008).

Glucocorticoids have been shown to exert specific effects on endometrial cells (Hoffman *et al.*, 1984; Bigsby, 1993; Jo *et al.*, 1993) but their role in endometrial physiology is not defined (Jabbour *et al.*, 2006). There are very limited data regarding GR expression in the endometrium. The GR is almost exclusively expressed in the stromal compartment including the endothelial and lymphoid cells (uterine Natural Killer cells) (Henderson *et al.*, 2003; Bamberger *et al.*, 2001). Expression of GR was low in glandular epithelium across the cycle however there was a significant increase in expression in decidual glands. There was high expression of GR in the endothelium in both functional and basal layers of the endometrium throughout the menstrual cycle and in first trimester decidua. Expression of GR protein increased across the cycle in the surface epithelium, from negligible expression in the proliferative and early secretory phases to weak

expression in the late secretory phase (McDonald *et al.*, 2006). It has been demonstrated that uterine Natural Killer (uNK) cells express GR (Henderson *et al.*, 2003). GR mRNA expression is not cycle-dependent as shown by the recent data on endometrial mRNA levels across the cycle (Vienonen *et al.*, 2004). GR mRNA was present in normal endometrium at low levels across the cycle and in similar amounts in the first trimester decidua (McDonald *et al.*, 2006).

Numerous studies have suggested roles for inflammatory markers, cytokines, prostaglandins and other agents in processes related to embryo implantation and endometrial shedding and repair, both pre and post menopausal (Salamonsen *et al.*, 2007; Henderson *et al.*, 2003; Lea and Sandra, 2007; Hickey *et al.*, 2005). Glucocorticoids could potentially influence these processes and hence a clear definition of the receptor expression and its effects are crucial to better understand these processes.

1.5.4 Ki-67 marker expression

The Ki-67 protein was originally defined by the prototype monoclonal antibody Ki-67 which was generated by immunizing mice with nuclei of the Hodgkin Lymphoma cell line L428 (Gerdes *et al.*, 1983). A detailed cell cycle analysis revealed that the antigen was present in the nuclei of cells in the G_1, S and G_2 phases of the cell division cycle as well as in mitosis. Quiescent or resting cells in the G_0 phase did not express the Ki-67 antigen (Gerdes *et al.*, 1984) (Figure 1.9). Because the Ki-67 antigen was present in all proliferating cells (normal and tumour cells), it became evident that the presence of this protein could constitute an excellent operational marker to determine the growth fraction of a given cell population (Scholzen and Gerdes, 2000).

Figure 1.9 Diagram depicting the cell cycle and the temporal expression of Ki-67 and Phosphorylated Histone-3 (PH3) markers

Since the Ki-67 antigen is expressed during several phases of the cell cycle e.g. G1, S, G2 and M (Gerdes *et al.*, 1984), counts of Ki-67 positive nuclei in paraffin sections are numerically far higher than the mitotic counts in the same sections, though the Ki-67 index is usually well correlated with the mitotic index. However, this correlation does not always hold. For example, in endometria of estrogenized macaques treated with either vehicle or antiprogestin for 2 weeks, the antiprogestin caused a dramatic suppression in the mitotic index but no suppression of the Ki-67 index (Slayden *et al.*, 2001a). Therefore, counts of Ki-67-stained cells failed to reveal that the antiprogestin treatment had suppressed estrogen-dependent proliferation. Presumably, the cells were blocked at a stage during which Ki-67 protein was continually expressed but from which cells could not progress through mitosis. In such cases, only the mitotic index accurately reflects the number of cells that complete the cell cycle (Brenner *et al.*, 2003b). Despite some of these drawbacks, Ki-67 remains a useful marker of cellular proliferation.

The Ki-67 expression correlates well with estrogen stimulation. The Ki-67 expression increases in the epithelial, glandular and stromal cells in the proliferative phase and decreases after ovulation in the secretory phase (Mertens *et al.*, 2002; Jones *et al.*, 1998; Maia *et al.*, 2004). Dahmoun and colleagues observed that the Ki-67 index increased in the stroma during the last 3 days of the secretory phase, parallel with an increasing progesterone receptor score and peaking at the onset of menstruation. These findings concur with high proliferation at the end of the menstrual cycle and high cell turnover during menstruation, suggesting the participation of stroma in the renewal process of endometrium (Dahmoun *et al.*, 1999). Furthermore, compared to normal endometrium, increased Ki-67 expression has been observed in endometrial polyps suggestive of progressive endometrial proliferative activity (Maia *et al.*, 2004). Ki-67 immunostaining is thought to be a satisfactory technique in evaluating the effects of hormone therapy even in postmenopausal endometrium (Darj *et al.*, 1995). In postmenopausal endometrium, Ki-67 immunostaining was significantly increased in stromal as well as glandular cells after estrogen-only treatment but these effects were clearly counterbalanced by the addition of Medroxyprogesterone Acetate (Klaassens *et al.*, 2006).

1.5.5 Phosphorylated Histone 3 (PH3) marker expression

Accurate measurement of cell proliferation in histological sections of endometrium is an important parameter in the assessment of hormone action, menstrual cycle stage and neoplastic growth (Brenner *et al.*, 2003b). These proliferative activities in endometrial cells have been studied in various ways including immunohistochemical analysis of cell cycle restricted proteins - proliferating cell nuclear antigen and the Ki-67 antigen (Hayama *et al.*, 2002). Direct counting of mitotic cells in haematoxylin and eosin (H&E)-stained sections is a time-consuming process that requires highly skilled observers (Hall and Levison, 1990). It is especially difficult to distinguish between cells in prophase and cells undergoing pycnosis or apoptosis in H&E-stained sections. Relatively high magnifications have to be used to distinguish the cycle phases and large numbers of cells must be counted to obtain statistical validity (Brenner *et al.*, 2003b). Therefore mitotic markers that can be easily studied are required and PH3 serves as a reliable marker for assessing cellular mitosis in various body tissues including the endometrium. The PH3 antibody has been used to identify chromosomes during mitosis in cultured cells (Brenner *et al.*, 2003b). It is expressed only in the M phase of the cell cycle (Chadee *et al.*, 1999) (Figure 1.9).

Eukaryotic cells must possess mechanisms for condensing and decondensing chromatin. Chromatin condensation is particularly evident during mitosis and cell death induced by apoptosis, whereas chromatin decondensation is necessary for replication, repair, recombination and transcription. Histones are among the numerous DNA-binding proteins that control the level of DNA condensation, and post-translational modification of histone tails plays a critical role in the dynamic condensation/decondensation that occurs during the cell cycle (Prigent and Dimitrov, 2003). Histone phosphorylation was first observed in the sixties (Gutierrez and Hnilica, 1967). Histone phosphorylation is believed to play a direct role in mitosis, cell death, repair, replication and recombination (Ito, 2007). During mitosis, histone H3 is phosphorylated at Ser10 in all eukaryotes (Prigent and Dimitrov, 2003) and with the development of an antibody against histone H3-phospho Ser10, it was established that there is a strong correlation between Ser10 phosphorylation and chromosome condensation during cell division (Hendzel *et al.*,

1997). However it has been suggested that Histone H3 phosphorylation is only required for initiation but not maintenance of mammalian chromosome condensation (Van Hooser *et al.*, 1998).

Endometrial epithelial cells and stromal cells proliferate briskly during the proliferative phase of the menstrual cycle, but their proliferation is less marked during the secretory phase. Since the anti-PH3 antibody has only recently been introduced, there are very limited data regarding PH3 expression in the endometrium. Brenner and colleagues evaluated the mitotic index and the PH3 expression in human and Rhesus macaque endometrium. In the human endometrium, the mitotic index and PH3 expression correlated well with increased evidence of mitosis in the proliferative phase and reduced expression in the secretory phase. In the macaque endometrium they further evaluated the PH3 expression in the functional and the basal layers after treatment with estrogen and/or progesterone. After treatment with estrogen, abundant PH3 positive cells were seen in the functional layer and the counts sharply decreased after addition of progesterone. However in the basal layer, the mitotic counts were very low during treatment with estrogen but they increased after treatment with progesterone suggesting that progesterone stimulates proliferation in the basal zone but suppresses it in the functional layer (Brenner *et al.*, 2003b). Narvekar and colleagues observed significant PH3 expression in the proliferative phase in the endometrial glands and stroma suggestive of active mitosis consistent with endometrial proliferation (Narvekar *et al.*, 2004). However PH3 expression was suppressed after treatment with mifepristone (Narvekar *et al.*, 2004). Compared to normal endometrium in proliferative phase, endometrium from PCOS women showed higher mitotic activity in the glands, whereas postmenopausal women had a significantly lower mitotic activity in the stroma. The mitotic activity in the stromal compartment (PCO group) and glands (postmenopausal group) was not significantly different from that in proliferative samples (Narvekar *et al.*, 2004).

1.5.6 Insulin like Growth Factor Binding Protein – I (IGFBP-I) expression

Insulin-like Growth Factor-I (IGF-I) and IGF-II are polypeptides that stimulate cellular proliferation and differentiation (Baxter, 1986). Endometrial stromal cells

produce Insulin-like growth factors I and II (IGF-I and IGF-II) as well as the high-affinity IGF binding proteins (IGFBPs), whereas epithelial cells and, in a lesser amount, also stromal cells contain cell membrane receptors for IGFs (Rutanen, 1998). The biological actions of IGFs are mediated through cell membrane receptors (Rechler and Nissley, 1985). In serum and some other media, IGFs are bound to binding proteins (BPS) (Baxter and Martin, 1989) and the BPS are believed to modulate the availability of the IGFs to their target cell thereby acting as regulators of IGF actions (Baxter and Martin, 1989; Elgin *et al.*, 1987; Busby *et al.*, 1988; Rutanen *et al.*, 1988; Ritvos *et al.*, 1988).

Although three distinct IGF binding proteins (IGFBPs) have been characterized in the human endometrium (Rutanen *et al.*, 1986; Giudice *et al.*, 1991), the most abundant is IGFBP-I (Rutanen, 2000). IGFBP-1 is synthesized and secreted by human secretory phase and pregnancy endometrium, and progesterone induces its synthesis in endometrial explant cultures (Rutanen *et al.*, 1986; Rutanen et al., 1985). The precise role of the IGFs and their BPS in the endometrium is not fully understood. Insulin-like growth factors, IGF-I, IGF-II, and IGF binding protein (IGFBP-1) appear to play major roles in endometrial development during the menstrual cycle and in the process of implantation. The mitogenic, differentiative and anti-apoptotic properties of these growth factors as well as their spatial and temporal expression in cycling endometrium suggest that they may participate in endometrial growth, differentiation, inhibition of apoptosis, and perhaps angiogenesis (Giudice *et al.*, 1998). IGFBP-1 is a major protein product of non-pregnant endometrium during the mid-late secretory phase and occurs in abundance in decidua. Its roles as an IGF-binding protein and as a trophoblast integrin ligand suggest that it may have multiple roles in endometrial development and in interactions between the decidua and the invading trophoblast (Giudice *et al.*, 1998). It is therefore studied as a marker of decidualisation. Estrogen stimulates IGF-I gene expression in the endometrium and IGF-I is assumed to mediate estrogen action. In contrast, progesterone stimulates the secretion of IGFBP-1 from the predecidualized/decidualized endometrial stromal cells in late secretory phase endometrium and pregnancy deciduas, thus counteracting the effects of the estrogen mediated stimulation of IGF-1 (Rutanen, 1998). The primary negative

regulator of IGFBP-1 expression is insulin, by inhibiting IGFBP-1 transcription. IGFBP-1 inhibits the receptor binding and biological actions of IGF-I in the endometrium and in cultured human trophoblastic cells. These findings support the view that the IGF system has autocrine and paracrine functions in the regulation of endometrial proliferation and differentiation (Rutanen, 1998). After treatment with the LNG-IUS, strong cytoplasmic staining for IGFBP-1 was detected in decidualized endometrium in women. Simultaneously oestrogen receptors were present, while progesterone receptors were hardly detectable in the endometrium by immunohistochemistry. The latter findings suggest that suppression of IGF-I action by IGFBP-1 may be one of the molecular mechanisms accounting for progestagenic and anti-oestrogenic effects of LNG-IUS in the endometrium (Rutanen, 2000).

1.6 Implantation

Despite multiple advances in assisted conception, the pregnancy rates continue to remain low. As a part of assisted conception treatment regimes, GnRH analogues and more recently GnRH antagonists are commonly used. Controlled ovarian stimulation leads to supraphysiological levels of ovarian steroids and hence concern has been raised as to the adverse impact of this on successful implantation (Nikas *et al.*, 1999; Papanikolaou *et al.*, 2005). There is some evidence suggesting endometrial histological advancement at the time of oocyte pick up in women treated with rFSH and GnRHantag (Kolibianakis *et al.*, 2002; Kolibianakis *et al.*, 2003). However there are limited data on the state of the endometrium during the crucial window of implantation. Although not clearly defined, the window of implantation is thought to extend from day 6 to day 10 after the luteinizing hormone (LH) surge.

In the endometrium, several molecules/factors that might affect implantation have been identified (Sharkey and Smith, 2003; Lessey, 1998) but so far there is limited understanding about the mechanisms involved. The actual process of implantation is thought to be an interaction between the endometrium and the embryo that is mediated by soluble growth factors (Yoo *et al.*, 1997; Bausero *et al.*, 1998), hormones, adhesion molecules (Horne *et al.*, 2001; Lessey, 2002a), the

extracellular matrix (ECM) (Jokimaa *et al.*, 2002), cytokines and prostaglandins (Kelly *et al.*, 2001). GnRHantag. may impact the processes of implantation through sex steroids or through direct effects via the GnRH receptors in the endometrium.

Sex steroids; estrogens (Lessey *et al.*, 2002b), progestogens (Lessey, 2003), and likely also androgen (Apparao *et al.*, 2002), are thought to play a vital but as yet not fully defined role in the complex mechanisms underlying endometrial development leading up to and after embryo implantation. These hormones act via their cognate receptors. After binding to the nuclear receptors, steroid receptors activate gene transcription. With the advent of microarray technology, the focus has shifted to trying to understand the endometrial gene profile in the mice (Wang and Dey, 2006) and even in humans (Kao *et al.*, 2002). Several genes have been recently identified and alteration in their expression is thought to have a major impact on implantation. Recent studies have analysed the gene expressions in physiological mid-luteal endometrium and in stimulated IVF/ICSI treatment cycles including in treatment cycles involving use of GnRH antagonists (Horcajadas *et al.*, 2008; Macklon *et al.*, 2008). These studies have described patterns of up-regulation and down-regulation of different sets of genes but it does appear that with further studies recognisable patterns of expression will become clearer. Attention can then be focussed on trying to establish the functional significance of these patterns of gene expression. The gene expression is partly thought to be under the control of estrogen and progestogens (Borthwick *et al.*, 2003). Further, it is recognised that elevated AR level during the window of implantation is associated with a decrease in a well-characterized biomarker of endometrial receptivity, the $\alpha_v\beta_3$ integrin (Apparao *et al.*, 2002).

The cellular and molecular expression of PR, ER and AR during the window of implantation in rFSH and GnRHantag treated endometrium has not yet been reported. Furthermore, the endometrial intracellular ligand availability and changes if any have not yet been evaluated in the context of embryo implantation.

1.7 Postmenopausal Endometrial Breakthrough Bleeding

Postmenopausal hormone replacement therapy (HRT) use is a common cause of breakthrough bleeding (BTB). It can occur in upto 60% of HRT users. Most

women will undergo investigations such as outpatient biopsy, ultrasound or hysteroscopy but in the majority, no cause is found (Elliott *et al.*, 2003). Majority of women on HRT, use a continuous combined preparation of estrogen and progestogen; the so-called bleed free HRT. In those on combined, sequential HRT regimens, both breakthrough and prolonged withdrawal bleeding contain morphological and presumably molecular alterations of the endometrium similar to those seen and described in oral contraception (OC) users. The histology of the endometrium exposed to combined, continuous HRT (cc-HRT) regimens is more variable than that of OC users. This is probably due to the lower dose of sex steroids used in the former than in the latter regimens (Ferenczy., 2003).

The exact cause of HRT related BTB is unknown. Changes in endometrial blood vessels, uterine natural killer cells, matrix metalloproteinases (MMP's) and their tissue inhibitors (TIMPs) have all been studied in women with BTB on HRT (Hickey *et al.*, 2005; Hickey *et al.*, 2006; Hickey *et al.*, 2008). There are few data on the steroid receptor expression in postmenopausal endometrium. Dahmoun and colleagues obtained endometrial biopsies from postmenopausal women before and 12 months after using cc-HRT. They observed that the PR expression in epithelium and stroma remained unchanged. After treatment with HRT, ER expression in epithelium and stroma was reduced and Ki-67 index in stroma was increased. They proposed that the increased stromal proliferation may be of importance for stromal support of the veins and for decreasing breakthrough bleeding during HRT. The increased stromal proliferation, as well as the decreased ER expression both in epithelium and stroma, could be an effect of progesterone (Dahmoun *et al.*, 2004).

1.8 Effects of Levonorgestrel – releasing Intra Uterine System on the Endometrium

The levonorgestrel – releasing intrauterine system (LNG-IUS) affords effective contraception and reduces menstrual blood loss and dysmenorrhoea. The system releases levonorgestrel at a slow rate of 20μg over 24 hours into the uterine cavity (Luukkainen *et al.*, 1990). Initially it was introduced as a contraceptive but now heavy uterine bleeding is a primary indication for use of the LNG- IUS in Europe (Jensen, 2002). The LNG-IUS also provides endometrial protection in combination

with oestrogen as a mode of post-menopausal hormonal replacement (Jensen, 2002). The main side effect of steroid contraception including LNG-IUS is irregular or unscheduled breakthrough bleeding. This leads to discontinuation in a proportion of users (Möller *et al.*, 2005). The mechanisms underlying BTB remain poorly understood but there is likely involvement of angiogenic factors (Roopa *et al.*, 2003), Matrix Metallo-Proteinases (MMP) activation (Skinner *et al.*, 1999) and altered haemostatic factors that result in increased endometrial vessel fragility (Hickey and Fraser, 2002). The presence of this side effect in up to 53% of women after 3 months' use of LNG-IUS provides justification to develop compounds that are more effective but with an improved side effect profile. Intra-uterine delivery of the Progesterone Antagonist (PA) ZK230211 is one such compound under evaluation.

The endometrium is a target tissue for steroid hormones and PAs. The intrauterine delivery of an androgenic progestogen, LNG, induces a dramatic transformation of the endometrium characterised by extensive decidualisation (Critchley *et al.*, 1998a). There is a reduction in the cellular proliferative activity as evidenced by the decrease in immunoreactivity of the cell proliferation marker Ki-67. Additionally, the immunostaining scores of epithelial Progesterone Receptor (PR) and stromal PR have been reported to be significantly lower after 6 months of LNG-IUS use and there is a non-significant decline in the Estrogen Receptor (ER) immunoreactivity in both the epithelial and stromal cells (Hurskainen *et al.*, 2000). In the normal cycle, Androgen Receptor (AR) has been spatially localised to the endometrial stroma with up-regulation in the estrogen dominated proliferative phase and down regulation in the late secretory phase. The presence of LNG-IUS was associated with minimal AR immunoreactivity in the stromal compartment and any duration of treatment with LNG-IUS caused a significantly lower production of AR mRNA when compared to the levels in proliferative phase (Burton *et al.*, 2003). Table 1.1 summarises the endometrial effects on LNG-IUS (Adapted from Guttinger and Critchley, 2007).

Table 1.1 Endometrial effects of Levonorgestrel releasing – intra uterine system

Morphology	Sex steroid receptors	Local factors	Intracrinology	Other factors
• Endometrial atrophy • Decidualisation • Altered spiral artery formation • Superficial vessels dilated and walls thinned	• ER↓ • PR↓ • AR↓	• Cytokines ↑ • Prostaglandins ↑ • VEGF ↑ • MMPs ↑ • Decidualisation markers ↑	• 17β-HSD2 ↑ • Estradiol ↓ • Estrone ↑	• Leucocyte infiltration

The angiogenic factors in the endometrium are of great interest in relation to understanding the mechanisms of action of the LNG-IUS. This is particularly relevant since BTB is the main side effect responsible for patient requests for early removal of the IUS (Möller *et al.*, 2005). Histological sections of endometrium from women using most of the long term progestin-only contraceptive (LTPOC) options display abnormally enlarged blood vessels at bleeding sites. A paradoxical observation is the reported trend toward reduced endometrial perfusion in LTPOC users. Basic laboratory studies point to local changes in the endometrial microvasculature following exposure to long acting progestogen, to be a contributory cause for BTB. It has been suggested that the mechanisms controlling endometrial angiogenesis could be altered with the use of long acting progestogen contraceptives. A recent study showed that there was an increased mRNA expression of the angiogenic growth factor VEGF in the endometrial tissues of women exposed to LNG-IUS. The effects of VEGF seem to be exclusively on the vascular endothelial cell. The study also noted a positive correlation between endometrial VEGF levels and the number of bleeding/spotting days (Roopa *et al.*, 2003).

1.9 Effects of Progesterone Antagonists on the Endometrium

Progesterone antagonists (PAs) are compounds that bind to the progesterone receptor and inhibit P-initiated gene transcription. With several compounds synthesized and characterised, the PA's have been further classified on the basis of their molecular actions. Some PA's have a partial agonistic/antagonistic activity on the Progesterone receptor – the so called Progesterone Receptor Modulators (PRM's), and some are thought to be pure antagonists that effect a progesterone blocking action by binding to the PR. Since the introduction of RU486 (mifepristone), the first potent PA synthesized, studies have shown that PA's can induce amenorrhoea, suppress ovulation, inhibit endometrial proliferation in combination with oestrogen therapy and act as a contraceptive (Baird *et al.*, 2003a; Baird *et al.*, 2003b).

With such widespread effects the PA's have potential applications in the treatment of heavy menstrual bleeding, fibroids, endometriosis, as a combination hormone replacement therapy, in breast cancer and as a contraceptive.

The type III PA ZK230211 is a pure PA that does not display any PR agonistic activity in-vivo or in-vitro. Since this is a relatively new compound (Fuhrmann *et al.*, 2000), virtually all the available data on local mechanisms of action come from animal studies. It is a highly potent PA with reduced anti-glucocorticoid activity. The effects of ZK230211 are thought to be species specific. For example in rodents and guinea pigs it did not inhibit estrogen-induced uterine growth or endometrial proliferation but enhanced it instead (Chwalisz *et al.*, 2000). These effects were not seen in the primate uterus. In the non-human primate uterus, subcutaneously administered ZK230211, chronically at relatively low doses, inhibits mitotic activity of endometrial epithelium and stromal compaction in spayed and intact macaques indicating that it blocked both progesterone and estrogen responses in the endometrium. Nevertheless there was no inhibition of the estrogen stimulation of oviductal and vaginal growth or differentiation in monkeys (Slayden *et al.*, 2001a). Since the effect is specific to the primate endometrium, the term 'endometrial antiproliferative effect' has been suggested (Brenner *et al.*, 2002). A dose finding study tested different doses of ZK230211 given daily, subcutaneously for 60 days in naturally cycling rhesus macaques. Doses of 0.016mg/kg body weight and 0.05mg/kg suppressed all ovulation and menstruation cycle to approximately 50 days. However circulating estradiol concentrations were never suppressed below normal, follicular phase concentrations at any dose. Therefore if it is used to block menses, it is unlikely to cause any associated symptoms of estrogen deprivation such as, hot flushes, vaginal atrophy or loss of bone density.

Following treatment with ZK230211, menstruation and ovulation were blocked in the non-human primate. The endometrial immunohistochemistry displayed interesting features. Treatment with higher doses (0.016 and 0.032mg/kg) blocked progesterone suppression and led to increased ERα, PR and Ki-67 immnuostaining in endometrial glands and stroma (Slayden *et al.*, 2001a). In general, at least with acute, short-term administration, PAs have been consistently shown to upregulate ER and PR in various tissues of the female reproductive tract in all species. This is

believed to be due to its antagonism of the PR. The action of PAs on the endometrial stromal cell is poorly understood. It has been suggested that down regulation of stromal growth factors may represent an important mechanism contributing to the endometrial antiproliferative effects of PAs.

Breakthrough Bleeding, a common side effect with chronic progestin therapy, was not observed with ZK 230211. In fact prolonged amenorrhoea lasting for many months frequently occurred after treatment with ZK230211 (Slayden *et al.*, 2001a). The vascular features observed after prolonged treatment were quite striking periarteriolar degeneration of endometrial spiral arteries leading in turn to their atrophy. The mechanism of this effect is poorly understood but local vasoactive factors including VEGF may play an important role (Chwalisz *et al.*, 2000).

CHAPTER 2

Hypothesis and Aims of Thesis

2 Hypothesis and Aims of Thesis

Endometrium is a vital steroid responsive target tissue that is involved in various functions such as menstruation, embryo implantation and after menopause, the cessation of cyclical shedding and repair. Previous sections in this thesis present the current understanding of the sex steroid receptor profile in the endometrium in relation to the above mentioned physiological events. However as is evident from the discussion so far, a clear understanding of the complex set of events in each situation is lacking.

In relation to embryo implantation, there are some available data regarding the endometrial sex steroid receptor expression during the putative window of implantation. However with the advent of assisted conception technologies and with the very significant hormonal manipulation incumbent in these treatment regimes, it is possible that the hormonal manipulation would have an impact, probably adverse, on the mid-luteal endometrium. GnRH antagonists have been introduced in IVF/ICSI treatment regimes within the last two decades. These agents do lead to significant hormone fluctuations within a short period of time and some studies have raised concerns about reduced pregnancy rates. Till date the impact of GnRH antagonists on the mid-luteal endometrium has not been adequately evaluated. The effects of GnRH antagonists could be mediated directly through the GnRH receptor which has been localised in the endometrium or they may be mediated indirectly via the steroid receptors or indeed through an alteration in the intracrine modulation thereby affecting ligand availability within the endometrial cell. The key enzymes involved in the intracellular ligand availability are the 17β-hydroxysteroid dehydrogenases and the 3β- hydroxysteroid dehydrogenases.

With progressive longevity, women spend increasing number of years post menopause. For a significant proportion of women, menopause brings with it troublesome complaints *inter alia* vasomotor symptoms and irregular vaginal bleeding. HRT is commonly used to alleviate these troublesome symptoms. All currently available HRT regimes have a common side effect – breakthrough bleeding. The mechanisms of HRT related breakthrough bleeding are ill understood. The steroid receptor expression at the time of a bleeding episode may

provide clues regarding the influence of HRT on human endometrium and especially its relation to HRT related breakthrough bleeding.

Menstruation is a regular and frequent cyclical event in the female reproductive life. Progestins and especially the levonorgestrel intra-uterine system (LNG-IUS) are widely used in the treatment of menstrual problems. However as with other progestins, breakthrough bleeding is a common side effect associated with the use of LNG-IUS leading to discontinuation of use by some women. In comparison, progesterone antagonists (PA) have shown encouraging results. They reduce menstrual blood loss without leading to BTB. In primate studies, systemic administration of a PA ZK230211 led to suppression of ovulation and menstruation. Intra-uterine release of ZK230211 had an antiproliferative effect on primate endometrium. Thus intrauterine release of PAs, especially ZK230211, may make them useful in hormone therapy for various gynaecological indications in humans. Specifically, endometrial suppression by means of PA-releasing IUS might be effective in the treatment of heavy or prolonged uterine bleeding. Moreover, an AP-IUS is likely to convert the endometrium into a non-receptive state, which may be utilized in the development of novel contraceptive strategies.

Based on available data on the above conditions, the following **hypotheses** are proposed:

1. Endometrial exposure to recombinant FSH and a GnRH antagonist alters steroid receptor and the sex steroid hormone metabolizing enzyme expression, thereby influencing the intracellular availability of sex hormones within the endometrium. These alterations may contribute to reduced pregnancy rates seen in women treated with recombinant FSH and a GnRH antagonist as a part of IVF/ICSI treatment.
2. All HRT preparations have a common side effect – breakthrough bleeding. In the pre-menopausal state, progesterone use leads to similar side effects. Continuous combined HRT leads to progestational effects on the endometrium. Hence, mechanisms of breakthrough bleeding should be similar in pre-menopausal long term progestin users and in postmenopausal continuous combined HRT users. The steroid receptor expression should

therefore mimic the changes seen after pre-menopausal long term progestin use.

3. Intrauterine delivery of progesterone antagonists causes endometrial antiproliferative effects and amenorrhoea in primate models. With many commonalities between primate and human endometrium, similar changes should occur in human endometrium after long term administration of AP-IUS.

The **aims** of this thesis thus were:

1. To study the midluteal phase endometrium during the putative window of implantation after treatment with a rFSH and a GnRH antagonist as used in an IVF/ICSI treatment cycle. The aim was also to determine the endometrial intracrinology in relation to expression of sex-steroid receptors and steroid metabolizing enzymes during the putative window of implantation.
2. To investigate whether a relationship exists between endometrial steroid receptor expression and bleeding patterns in HRT users.
3. To evaluate the endometrial effects of intrauterine release of the PA ZK230211 versus progestin LNG in women with complaints of heavy or painful menstruation with a view to assess feasibility of clinical use.

CHAPTER 3

General Methods

3.1 Human Endometrial Tissue Collection

Endometrial tissue samples were obtained from women attending the gynaecology departments of three hospitals for the respective studies presented in this thesis. All women provided written informed consent and all studies were approved by the local institutional ethics committees.

3.1.1 Endometrial specimens from women exposed to rFSH and GnRHantag

Women who took part in this study were recruited in the Royal Infirmary of Edinburgh. For the entire study, endometrial tissue samples were obtained with a Pipelle® endometrial sampling device (Pipelle de Cornier, Laboratoire CCD, Paris, France) (Figures 3.1(a) and 3.1 (b)). After collection of endometrium, the tissue was fixed in 4% paraformaldehyde and then embedded in paraffin for immunohistochemical analysis. In addition, endometrium was also frozen at the point of tissue collection in liquid nitrogen and stored at -70°C. RNA was extracted from frozen endometrial tissue. All endometrial samples were histologically classified as per the Noyes criteria (Noyes *et al.*, 1950) to confirm the mid-luteal stage of the cycle. All studies on the endometrial tissue were carried out in the University of Edinburgh Centre for Reproductive Biology. The endometrial tissue samples were subjected to Immunohistochemistry and Quantitative Real Time – Polymerase Chain Reaction (QRT-PCR) as described in sections 3.2 and 3.3.

Figure 3.1 (a) Diagram of Pipelle® endometrial biopsy sampling

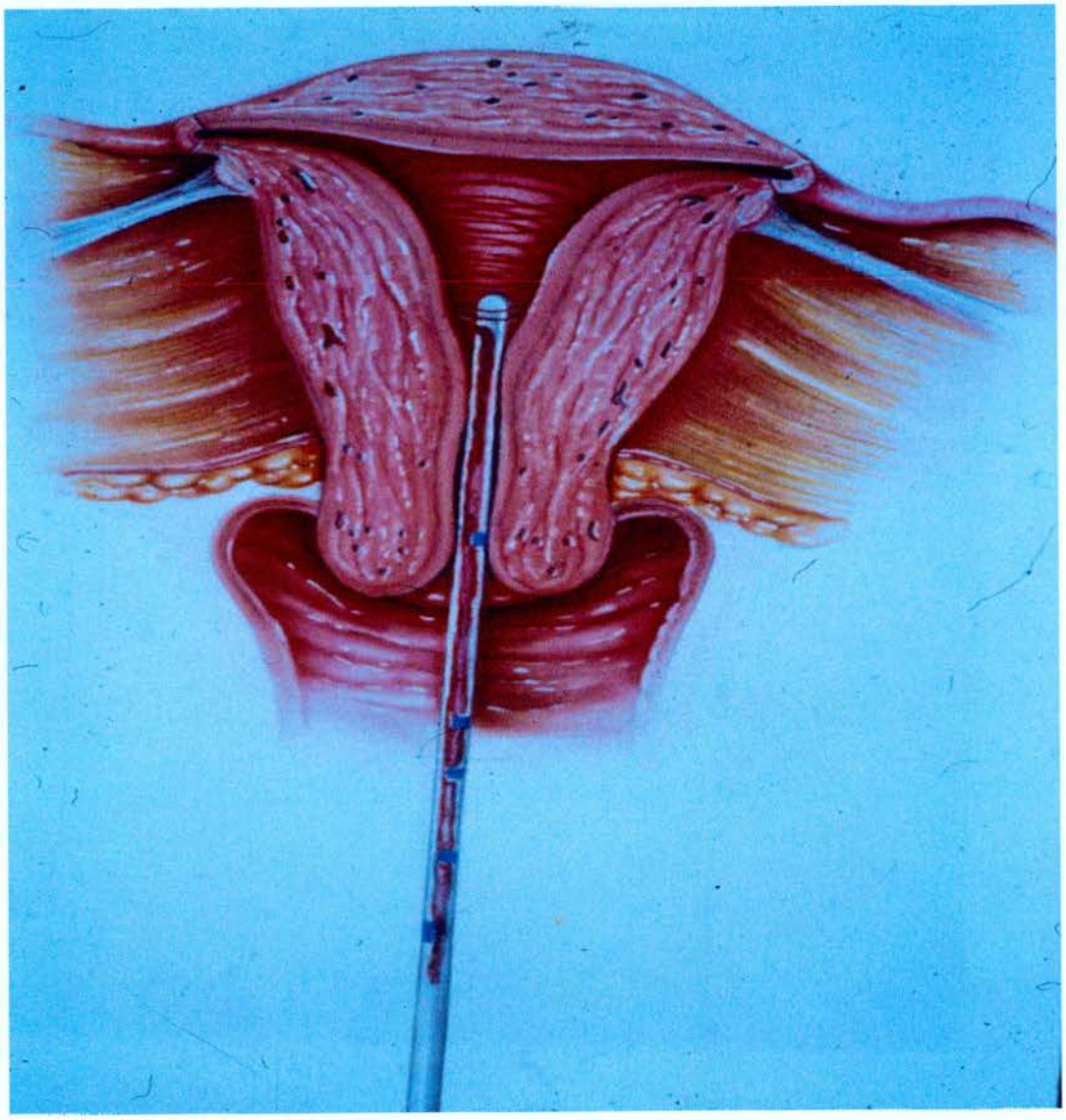
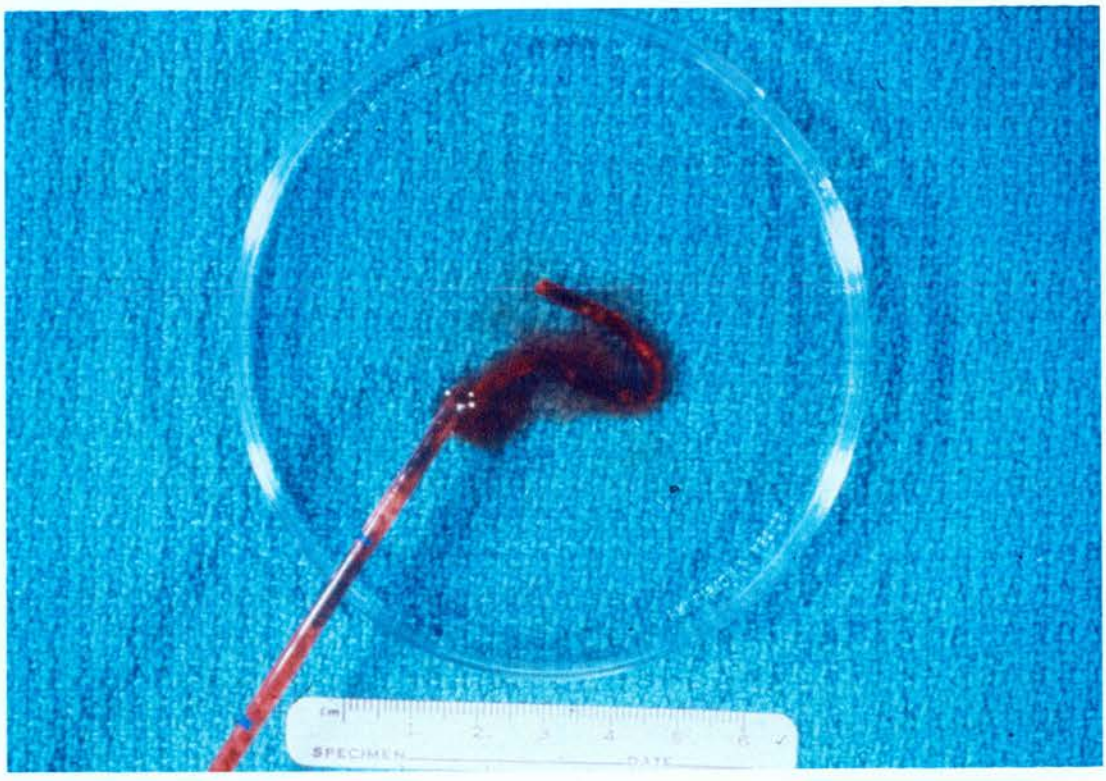


Figure 3.1 (b) Endometrial biopsy sample using the Pipelle® sampling device



3.1.2 Endometrial specimens from postmenopausal women in the presence or absence of Hormone Replacement Therapy

The endometrial samples for this study were obtained in the Menopause clinic at King Edward Memorial Hospital, Perth, Western Australia. Endometrial biopsies were obtained with the Pipelle® endometrial sampling device (Pipelle de Cornier, Laboratoire CCD, Paris, France). After collection, biopsies were fixed immediately in 10% formalin for 18 hours and tissue was then embedded in paraffin. Sections were cut at 5 μm. The sections were dried at 37°C overnight. All biopsies had a standard haematoxylin and eosin section submitted to an experienced histopathologist for classification according to Noyes criteria (Noyes *et al.*, 1950) and for identification of any pathological features. The tissue sections were subjected to immunohistochemical analysis (as described below) which was carried out in the University of Edinburgh Centre for Reproductive Biology.

3.1.3 Endometrial specimens from women using an Antiprogestin – Intrauterine System (AP-IUS)

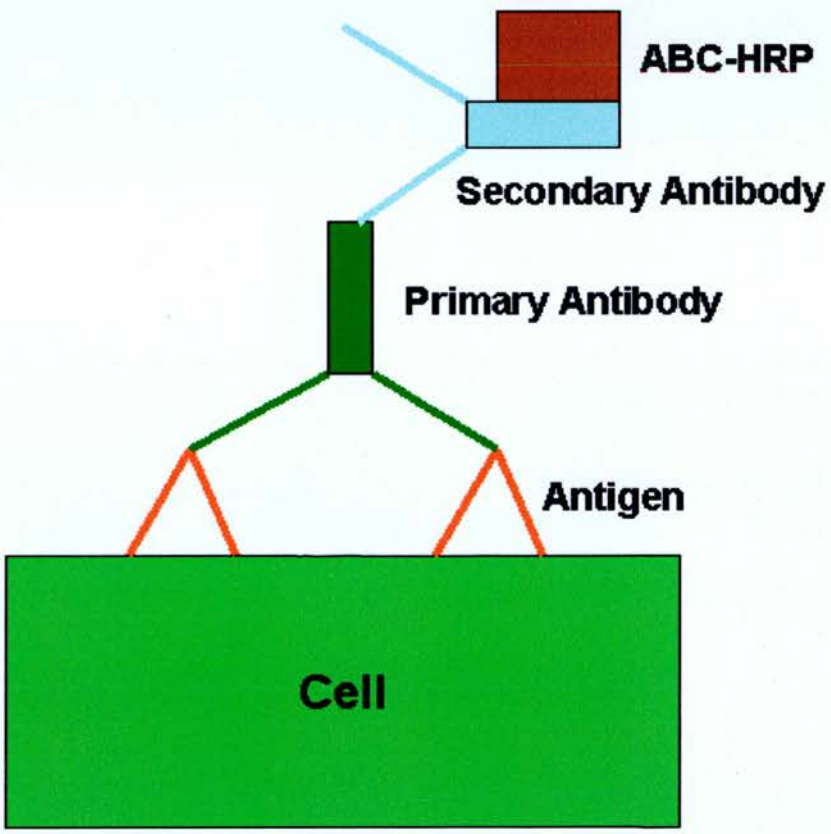
The endometrial tissue samples were obtained from women at the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital, Finland. Samples were obtained at the time of a hysterectomy. Following surgery and collection of specimens for routine pathological examination, separate samples of endometrium were collected from the fundal, mid-corpus and isthmic areas of the uterus for haematoxylin and eosin staining and immunohistochemistry (IHC). For the studies in this thesis, tissue samples from the fundus were used. The samples were frozen and pre-prepared paraffin blocks were submitted to the University of Edinburgh Centre for Reproductive Biology for immunohistochemical analysis. Immunohistochemistry experiments for all markers except the decidualization marker IGFBP-1 were conducted at the University of Edinburgh Centre for Reproductive Biology. Immunohistochemistry for IGFBP-1 was conducted in the University of Turku, Finland.

3.2 Immunohistochemistry

Immunohistochemistry is the process of localization of antigens or proteins in cells of a tissue section by the use of labelled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker. A chromogen, e.g. diaminobenzidine (DAB), used in all immunohistochemistry protocols included in this thesis, is an example of one such marker. A standard Avidin-Biotin Complex - Horseradish Peroxidase (ABC-HRP) method was employed in most of the studies. Briefly, the primary antibody binds specifically to the protein of interest and the biotinylated secondary antibody recognises and binds to the antigenic sites on the primary antibody. An avidin-biotin-HRP complex binds to the biotin on the secondary antibody and the marker DAB binds the HRP and is oxidised. This causes a visible colour change which results in a brown staining of the tissue sections at the sites of antibody interaction (Figure 3.2).

Figure 3.2 Diagrammatic representation of ABC-HRP immunohistochemistry method. Adapted from Dako UK Ltd. Handbook of Immunohistochemical staining methods 3rd edition, (Boenisch, 2001).

ABC-HRP = Avidin Biotin Complex - Horseradish Peroxidase



For studies in this thesis, immunohistochemistry (IHC) was performed for the localisation of several steroid receptors, steroid metabolizing enzymes and other markers. The steroid receptors studied included Estrogen Receptor α (ERα), Estrogen Receptor β (ERβ), Progesterone Receptor (PR), Androgen Receptor (AR) and the Glucocorticoid Receptor (GR). The steroid receptor metabolizing enzymes studied included 3β-Hydroxysteroid Dehydrogenase (3βHSD) and 17β-Hydroxysteroid Dehydrogenase Type 5 (17βHSD5). The currently available 3βHSD antibody recognizes both human 3βHSD1 and 3βHSD2 enzymes. No documented antibodies were commercially available for 17βHSD2 enzyme and hence no immunochemistry was performed for this enzyme. The proliferation markers studied included Ki-67 and Phosphorylated Histone 3 (PH3). The marker of endometrial decidualisation, Insulin like Growth Factor Binding Protein – 1 (IGFBP-1) was also studied with IHC.

All protocols were optimized to achieve the best conditions for immunostaining. The protocols are summarised below.

3.2.1 General Immunohistochemistry Protocol

Immunohistochemistry experiments were conducted by progressing through sequential steps in the following order.

- Slide preparation
- Antigen retrieval – was carried out in some experiments. This step is required to break the bonds created by fixation and to expose the antigen to allow antibodies to bind.
- Endogenous peroxidase block – the endogenous peroxidases need to be blocked to prevent excess background staining.
- Avidin-Biotin Block – endogenous avidin and biotin also need to be blocked to prevent excess background staining.
- Non-immune block – background staining was further prevented by the inclusion of a non-immune block.
- Primary antibodies – were diluted to the appropriate concentration determined by titration in the serum used for blocking. Where possible, the primary antibody was pre-absorbed against a blocking peptide for use as a

negative control. Alternatively, pre-immune serum or generic immunoglobulins from the same species at the same concentration as the primary antibody was used. Positive controls were tissue sections known to express high levels of the protein of interest.

- Secondary antibodies – biotinylated secondary antibodies raised against the immunoglobulins of the species in which the primary antibody was raised were diluted as described in the serum solutions used for the non-immune block.
- Tertiary antibody- The ABC-Elite detection system (Vector Laboratories) was used to amplify the signal from the biotinylated secondary antibody.
- Developing and counterstaining – slides were developed and staining was observed after applying 3, 3'-diaminobenzidine (DAB; Dako, Cambridge, UK).

An example of a general immunohistochemistry protocol is described below.

Paraffin block sections were cut to 5 microns thickness and were mounted on superfrost glass slides (BDH, Merck House, Poole, Dorset, England). Slides were dewaxed in histoclear for 10 min and then rehydrated in descending grades of alcohol (100% ethanol for 2 minutes, 95% ethanol for 2 minutes, 70% ethanol for 2 minutes) to distilled water (dH2O). The sections were washed in dH2O for 10 minutes. The slides were then washed in phosphate buffered saline (PBS, Sigma-Aldrich Ltd. Poole, Dorset, England) for 10 minutes.

After this, an antigen retrieval step was performed if required. Endogenous peroxidase activity was blocked. This was performed by incubating the sections in 3% hydrogen peroxide in dH2O for 10 min at room temperature. Tissue sections were then washed for 10 min in PBS. This was followed by 15 min incubation with avidin (Vector Laboratories Ltd, Peterborough, UK) at room temperature. After a rinse in PBS for 2 min, the sections were incubated with biotin (Vector Laboratories Ltd) for a further 15 min at room temperature. Following a 2-min wash in PBS, normal horse serum (NHS, Vectastatin, Vector Laboratories) was applied to each tissue section. This was followed by incubation for 20 min in a humidified chamber at room temperature. The excess serum was removed and the primary antibody was applied. The negative control for the primary antibody was

substituted with mouse immunoglobulin G (mIgG1, Sigma) or rabbit pre-immune serum at the same concentration. After the primary antibody incubation, the sections were washed between each stage for 10 min in PBS+Tween 20. The secondary antibody was then applied. To identify positive staining, the peroxidase substrate diaminobenzidine (Dako, Cambridge, UK) was used as chromogen for demonstration of epitope. Tissue sections were washed in dH2O and counterstained with Harris's haematoxylin (a non-specific purple nuclear stain), dehydrated in ascending grades of alcohol and mounted from xylene using pertex mounting medium. A similar protocol was used for immunostaining of all receptors. Specific incubation conditions for immunolocalization of each of the epitopes studied are described here and are summarized in sections 4.2, 5.2 and 6.2 respectively.

BOND® Automated Immunohistochemistry

For the study examining HRT exposed endometrium, the BOND® (BOND system, Vision Biosystems, UK) automated system was used for IHC. Separate IHC runs were carried out for each steroid receptor. An antigen retrieval step was carried out as described previously and then the tissue sections were placed in the BOND® automated IHC machine for the remainder of the steps in the protocol. The sections were then removed from the machine, washed in dH_2O and counterstained with Harris's Haematoxylin, a non-specific purple nuclear stain. They were then dehydrated in ascending grades of alcohol and mounted from xylene using pertex mounting medium.

3.2.2 Androgen Receptor (AR) Immunohistochemistry Protocol

AR IHC was performed on paraffin sections (5 microns in thickness). These were dewaxed in histoclear for 10 minutes and rehydrated in descending grades of alcohol to distilled water (dH_2O) as described in section 3.2. The sections were then washed in dH_2O, after which a pressure cook antigen retrieval step was performed. The sections were heated in 0.01M sodium citrate buffer (pH6) for 5 minutes at setting 2/high in Tefal Clipso pressure cooker, followed by 20 minutes incubation in the pressure cooker. Following a 10minute wash in 0.01M Phosphate Buffered Saline pH 7.4 (PBS, Sigma, Dorset, UK), endogenous peroxidase activity

was blocked by incubating the sections in 3% hydrogen peroxide in dH_2O for 10 minutes at room temperature. Sections were then washed for 10 minutes in PBS followed by 15 minutes incubation with avidin (Vector Laboratories Ltd, Peterborough, UK) at room temperature. Following a 2 minute wash in PBS, normal horse serum (NHS, Vectastatin, Vector Laboratories, Peterborough, UK) was applied to each tissue section and incubated for 20 minutes in a humidified chamber at room temperature. The excess serum was carefully blotted off and 70µl of a monoclonal mouse anti-AR antibody (Biogenex, CA, USA), at a dilution of 1:240 in PBS/1%BSA/0.1%Gelatin (Sigma, Dorset, UK) was applied. Coverslips were placed over the tissue sections to minimise antibody evaporation and the sections were incubated overnight at 4°C. The primary antibody was substituted with mouse immunoglobulin G (MigG1, Sigma, Dorset, UK) at a matched concentration (1:300), to the androgen receptor antibody for the negative control sections.

After the primary antibody incubation, the sections were washed between each stage for 10 minutes in PBS + Tween 20 (PBST). The sections were incubated with biotinylated horse-anti mouse IgG and then with an avidin-biotin peroxidase detection system, ABC-Elite (Vectastatin, Vector Laboratories Ltd, Peterborough, UK), both for 60 minutes at room temperature. To identify positive staining, the peroxidase substrate diaminobenzidine (DAB; Dako, Cambridge, UK) was applied to the tissue sections; this forms a brown precipitate on contact with the antigen-antibody complex. The sections were washed in dH_2O and counterstained with Harris's Haematoxylin, a non-specific purple nuclear stain, dehydrated in ascending grades of alcohol and mounted from xylene using pertex mounting medium.

3.2.3 Estrogen Receptor α (ERα) Immunohistochemistry Protocol

A protocol similar to AR IHC was used with the following exceptions. The sections were not pre-treated with avidin and biotin. Prior to the endogenous peroxidase block step, a microwave antigen retrieval step was necessary whereby the sections were heated in 0.01M sodium citrate buffer (pH6) for 10 minutes at high power followed by 20 minutes incubation in the oven. The primary antibody

used was monoclonal mouse anti-ERα antibody (Dako, Cambridge, UK), at a dilution of 1:400 in PBS. For the negative controls a 1:2400 dilution of MIgG1 in PBS was used. The primary antibody incubation was for 60 minutes at 37°C.

3.2.4 Estrogen Receptor β (ERβ) Immunohistochemistry Protocol

A protocol similar to AR IHC was used with the following exceptions. The sections were not pre-treated with avidin and biotin. The sections were washed in Tris Buffered Saline (TBS) instead of PBS. The sections were heated in 0.05M Glycine/0.01% EDTA buffer (pH8) for 7 minutes, setting 2/High in the pressure cooker. The endogenous peroxide block was carried out using 3% H_2O_2 in Methanol for 30 minutes at room temperature. A 1:5 dilution of normal rabbit serum in TBS + 5% BSA (NRS/TBS/BSA) was used for the non-immune block. The primary antibody used was monoclonal mouse anti-ERβ1 antibody (Serotec, Oxford, UK), at a dilution of 1:40 in NRS/TBS/BSA. For the negative controls, NRS/TBS/BSA was used. The secondary antibody was biotinylated rabbit anti-mouse IgG (Dako, Cambridge, UK) at a concentration of 1:500, prepared in NRS/TBS/BSA. ABC-Streptavidin (Dako, Cambridge, UK) was used in place of ABC-Elite.

3.2.5 Progesterone Receptor (PR) Immunohistochemistry Protocol

A protocol similar to the AR IHC was used with the following exceptions. The sections were not pre-treated with avidin and biotin. Prior to the endogenous peroxidase block step, a microwave antigen retrieval step was necessary whereby the sections were heated in 0.01M sodium citrate buffer (pH6) for 10 minutes at high power followed by 20 minutes incubation in the oven. The primary antibody used was monoclonal mouse anti-PR antibody (Novocastra, Newcastle, UK), at a dilution of 1:40 in NHS. For the negative controls a 1:800 dilution of MIgG1 in NHS was used. The primary antibody incubation was for 60 minutes at 37°C. Incubations with the secondary antibody and ABC were for 30 minutes only.

3.2.6 Glucocorticoid Receptor (GR) Immunohistochemistry Protocol

A protocol similar to that for AR IHC was used. The only exception was that the primary antibody used was the monoclonal mouse anti-GR (Novocastra, Newcastle, UK), diluted 1:40 in a 1:5 dilution of normal rabbit serum (Diagnostics Scotland) in PBS + 5%BSA (NRS/PBS/BSA) and a 1:320 dilution of MigG1 for negative controls.

3.2.7 3β-Hydroxysteroid Dehydrogenase (3βHSD) Immunohistochemistry Protocol

A protocol similar to AR IHC was used with the following exceptions. No antigen retrieval was performed. The primary antibody used was a polyclonal rabbit anti-3βHSD (Professor J I Mason, University of Edinburgh), at a dilution of 1:500 in NGS/PBS/BSA. The 3βHSD rabbit polyclonal antibody was raised against recombinant human 3βHSD2 and recognizes both human 3βHSD1 and 3βHSD2 with similar affinity (McDonald SE, Mason JI, Critchley HOD; unpublished observations). For negative controls, pre-immune serum at a dilution of 1:500 in NGS/PBS/BSA was used.

3.2.8 17β-Hydroxysteroid Dehydrogenase Type 5 (17βHSD5) Immunohistochemistry Protocol

A protocol similar to AR was used with the following exceptions. The primary antibody used was monoclonal mouse anti-17βHSD5 (a generous gift from Professor Trevor Penning, University of Pennsylvania, USA) at a dilution of 1:200 in NHS/PBS/BSA. For negative controls, a 1:300 dilution of MIgG in NHS/PBS/BSA was used.

Although a mouse monoclonal antibody against human 17βHSD2 was used in an earlier study (Burton *et al.*, 2003), neither this nor any commercial 17βHSD2 antibody were currently available and hence no immunochemistry was performed for this enzyme.

3.2.9 Ki-67 Immunohistochemistry Protocol

A protocol similar to the AR IHC was used with the following exceptions. Prior to the endogenous peroxidase block step, a microwave antigen retrieval step was necessary whereby the sections were heated in 0.01M sodium citrate buffer (pH6) for 10 minutes at high power followed by 20 minutes incubation in the oven. The primary antibody used was monoclonal mouse anti-Ki67 antibody (Novocastra, Newcastle, UK), at a dilution of 1:50 in PBS. For the negative controls a 1:500 dilution of MIgG in PBS was used. Incubations with the secondary antibody and ABC were for 30 minutes only. ABC-HRP was used in place of ABC-Elite.

3.2.10 Anti-Phospho-Histone 3 (PH3) Mitosis Marker Immunohistochemistry Protocol

A protocol similar to AR IHC was used with the following exceptions. The sections were not pre-treated with avidin and biotin. The primary antibody used was a rabbit anti-phospho-Histone (H3, Upstate Biotechnology, Buckingham, UK) antibody at a dilution of 1:1000 in normal goat serum (Vector Laboratories Ltd, Peterborough, UK). For the negative controls, a 1:1000 dilution of rabbit IgG (rIgG) (Vector Laboratories Ltd, Peterborough, UK) was used. The secondary antibody was biotinylated goat anti-rabbit IgG (Vector Laboratories Ltd, Peterborough, UK). ABC-HRP (Vectastatin, Vector Laboratories Ltd, Peterborough, UK) was used in place of ABC-Elite.

3.2.11 Insulin like Growth Factor Binding Protein – 1 (IGFBP-1) Immunohistochemistry Protocol

This part of the study was carried out in the Department of Pathology, University of Turku, Turku, Finland. The protocol used for IGFBP-1 immunostaining was as described previously (Pekonen *et al.*, 1992).

A protocol similar to the AR IHC was used with the following exceptions. The sections were pre-treated with avidin and biotin using the Vectastatin ABC-Kit (Vector Laboratories Inc., Burlingame, CA). The primary antibody used was monoclonal mouse anti-IGFBP-1 antibody (MAb 6303, Medix Biochemica, Kauniainen, Finland) at a dilution of 1:1000 in PBS containing 0.5% Tween. For

the negative controls a 1:1000 dilution of MIgG1 was used. The primary antibody incubation was for 60 minutes. Incubations with the secondary antibody and ABC were for 60 minutes. To identify positive staining, the peroxidase substrate 3-amino-9-ethylcarbazole (10mg/ml; red stain) in acetate buffer, pH5, and containing 0.03% hydrogen peroxide was applied.

3.2.12 Optimization of Immunohistochemistry Protocols

To ensure valid results from Immunohistochemistry experiments, optimization of immunostaining protocols is a vital step. Local immunostaining protocols have been optimized previously by other members of this laboratory prior to the start of these studies. For optimization experiments, all antibodies were tested individually at a range of dilutions and with different antigen retrieval conditions to determine the protocol that gave the least background and highest specific signal before additional optimization of double staining conditions (Henderson *et al.*, 2003).

To ensure validity of results in each run, positive controls were included in each immunostaining run. Endometrial tissue known to be positively stained for the respective receptor types was used as positive control. The positive control slides were stained along with the study slides and before analyzing the biopsies included in the respective study, the positive control slides were checked to determine appropriate staining.

Negative controls were also included in each run. These were stained by omitting the primary antibody or by replacing the primary antibody with normal serum preferably from the same species as the primary antibody. Absence of staining in the negative control would confirm the specificity of the antibody.

3.2.13 Immunohistochemical Analysis and Statistics

Throughout the studies included in this thesis, protein expression has been studied in 5 endometrial compartments i.e. Glands, Stroma, Surface Epithelium, Vascular Endothelium and in Perivascular cells. It is considered that perivascular cells are smooth muscle cells (rather than stromal cells), with separate functions and hence they are assessed independently of the adjacent stromal cells.

A semi-quantitative scoring system was utilised for all IHC experiments. In examining the slides, immunostaining scores were allocated on the basis of staining intensity. Examination of the slide was started at a low magnification to get a sense of overall staining across the slide. Then moving to a higher magnification the analysis focussed on the part of the slide representative of the overall staining. Staining intensity in each compartment was evaluated. Each observer scored the slides independently and then the scores were compared. Where there was agreement in scoring, these were accepted as the final scores. Where observers differed, a third observer, who was familiar with this scoring method, was asked to see the slides and allocate scores. The score that was allocated by 2 out of 3 observers was taken as the final score.

The immunostaining intensity and localization of epitopes in whole tissue sections was assessed in a semi-quantitative manner on a four point scale: 0 = no staining; 1 = mild/minimal immunostaining; 2 = moderate immunostaining and 3 = intense immunostaining. All tissue sections were scored blind by at least two observers. Because of the discontinuous nature of the semi-quantitative IHC data, the non-parametric Mann–Whitney test was performed. Where comparisons between groups were required, Dunn's multiple comparison correction was then applied to the *p* values from each set of tests. Results with a p value <0.05 were considered to be significant differences.

This scoring system has been previously validated in a study comparing quantitative and semi-quantitative scores (Wang *et al.*, 1998). In that study a high correlation with a regression coefficient of 0.963 was found between the two methods. This provides a high level of confidence in the semi-quantitative scoring system and since it is much less time consuming than quantitative scoring methods, the former was preferred in all the studies included in this thesis.

3.3 Real Time Quantitative Reverse Transcription – PCR (QRT-PCR)

3.3.1 RNA extraction

Endometrial tissue was collected either with a Pipelle® sampler or at hysterectomy as described in section 3.1. Tissue was immediately frozen at -70°C. Endometrium was homogenized and extraction was performed as detailed below. The tissue was handled in such a manner to minimize possibility of RNAse contamination. All instruments and disposables were certified RNAse free and sterile or were autoclaved prior to use.

For RNA isolation, Trizol reagent (Invitrogen Life Technologies Ltd, Paisley, UK) was used according to the manufacturer's instructions. This reagent is a monophasic solution of phenol and guanidine isothiocyanate. During the process of sample homogenization, Trizol maintains the integrity of the RNA while disrupting cells and dissolving cell components. Chloroform is added followed by centrifugation and this separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase and after transferring the aqueous phase, RNA is recovered by precipitation with isopropyl alcohol.

Using a hand-held homogenizer, 50-100mg of tissue was homogenized in 1ml of Trizol reagent. Each homogenized sample was placed in a phase-lock gel heavy tube and incubated for 5 minutes at 15-30°C to permit the complete dissociation of nucleoprotein complex. Chloroform (0.2 ml per 1 ml of Trizol reagent) was added. The sample tubes were capped, mixed by inversion for 15 seconds and incubated at room temperature for 3 minutes. The samples were centrifuged at no more than 13000 rpm for 15 minutes at 4°C for separation of the mixture into two portions i.e. the aqueous RNA containing phase above and the DNA/protein containing phase below the gel in the phenol red layer.

The top portion, the aqueous phase, was transferred to 2 ml eppendorf tube. Then 0.5 ml of 100% isopropyl alcohol per 1 ml of Trizol reagent used for the initial homogenization was added to allow precipitation of the RNA from the aqueous phase. The samples were then incubated at room temperature for 10 minutes and centrifuged at no more than 13000 rpm for 10 minutes at 4°C. The resulting supernatant was removed leaving a pellet containing the RNA precipitate. The

RNA pellet was washed once with 75% ethanol (by adding at least 1 ml of 75% ethanol per 1 ml of Trizol reagent used for the initial homogenization) mixing the samples by vortexing. The samples were then centrifuged at 7500 rpm for 10 minutes at 4°C. Finally, the RNA pellet was briefly air dried and then re-suspended in RNA storage solution (Ambion, Austin, Texas, USA) and stored at -80°C until required for Taqman PCR analysis.

3.3.2 RNA Quantitation

Optical density measurements were performed using the Genequant machine to quantify the concentration of RNA. This machine automatically calculates the concentration of RNA. A 1:10 dilution of RNA was made by dilution of 2 µl of RNA in 8 µl of nuclease free H_2O. An optical density ratio at a wavelength of 260nm to a wavelength of 280nm (260:280) was calculated. The sample was only considered sufficiently pure for use in the studies if the ratio was greater than 1.6. The concentration was then recorded.

3.3.3 Reverse Transcription

The reverse transcription polymerase chain reaction (RT-PCR) is a sensitive method for the detection of low-abundance mRNA. RT-PCR assays are the most common method for comparing mRNA levels in different sample populations (Orlando *et al.*, 1998). The first step in an RT-PCR assay is the reverse transcription of the RNA template into cDNA. This is done since RNA cannot serve as a template for PCR. The cDNA is exponentially amplified in a PCR reaction. A further advantage of the RT step being carried out separately from the PCR step is to facilitate the storage of the pool of cDNA for later use.

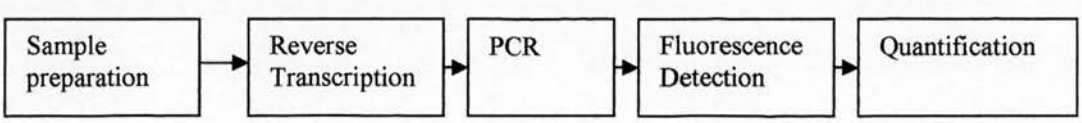
The genomic DNA was removed by subjecting the RNA to DNAse treatment. 1µg of each RNA was added to the individual mixtures containing 1µl of DNAse buffer and 1µl of DNAse with the remaining volume being made up to 10 µl with nuclease free water. The mixture was incubated at room temperature for 15 minutes at which point 1µl of 25 mM EDTA was added to stop the digestion reaction. Then the mixture was incubated on a PCR block for 5 minutes at 99°C to denature the DNAse. The mixture was then cooled on ice and samples were given a pulse

centrifuge to collect any sample condensed on the eppendorf wall. Samples could then be stored at -80°C until required for cDNA preparation.

The reverse transcription (RT) reaction was performed as described previously (Henderson *et al.*, 2003; McDonald *et al.*, 2006). The RT-PCR reaction is performed in a 10µl volume of reaction solution. 2 µl of DNAse treated mixture containing 200 ng of RNA or RNA stock was diluted in nuclease free water to prepare a concentration of 100ng/ µl from which 200ng (2 µl) of each sample RNA, including a positive control, were added into 8 µl of master mix solution. The master mix solution was made up of: 1xTaqman RT buffer, magnesium chloride (5.5 mmol/l), deoxyNTPs, random hexamers (2.5 µmol/l), multiscribe reverse transcriptase (1.25 IU/ µl), RNAse inhibitor (0.40 IU/ µl) and nuclease-free water (reagents from Applied Biosystems, Cheshire, UK). Each tube was mixed by vortexing, pulse centrifuged and over-layered with mineral oil. PCR reactions were carried out on a Hybaid Omn-E thermal cycler (Hybaid, Ashford, UK). The RT reaction was conducted at 25°C for 60 min, 48°C for 45 min and 95°C for 5 min for one cycle. An RT-negative control had template RNA but no multiscribe enzyme included, and an RT H2O had template RNA replaced by nuclease-free water. Negative controls were included in every run. The samples were then stored at -20°C.

3.3.4 Real Time RT-PCR

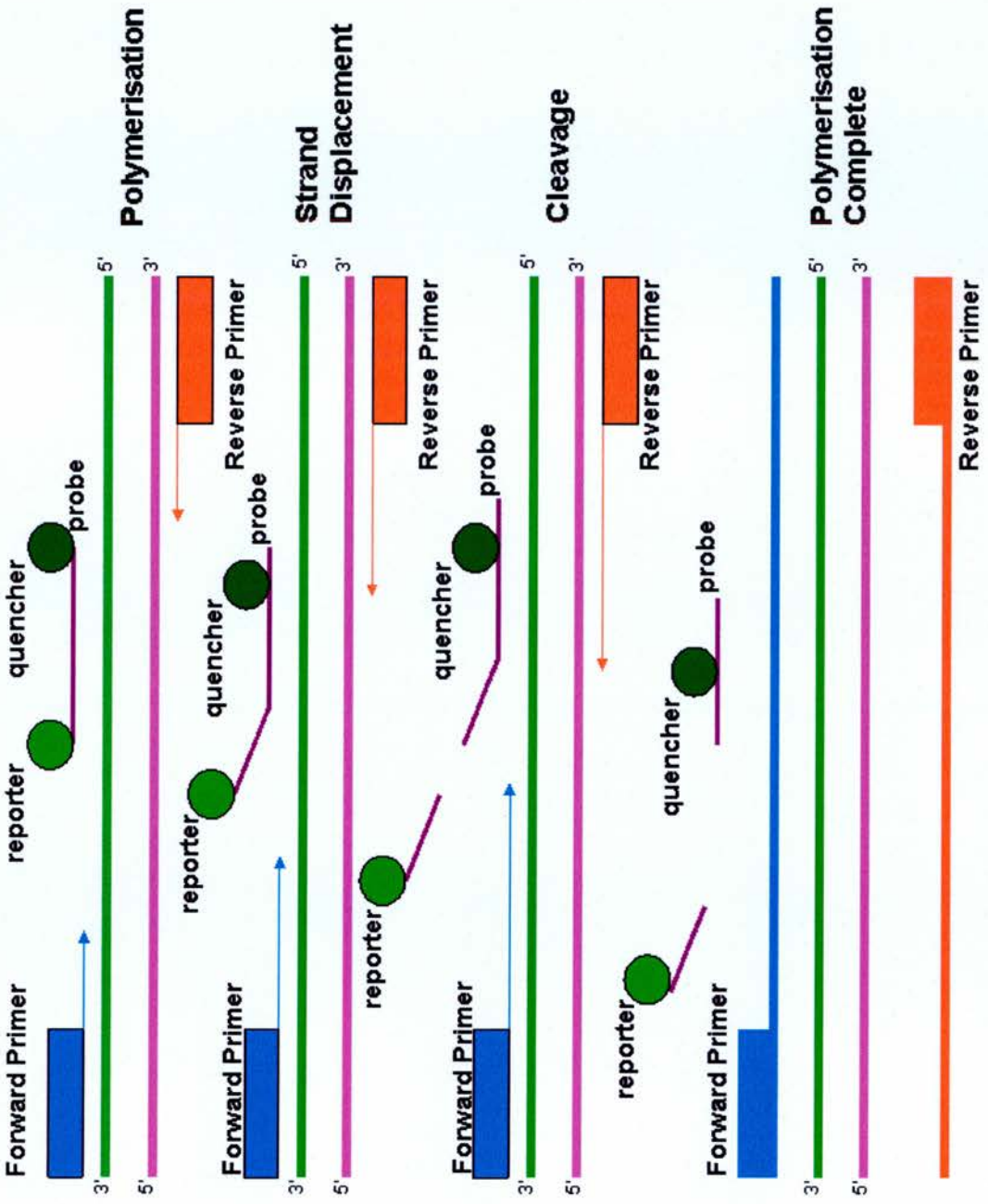
The Taqman assay (Perkin-Elmer-Applied Biosystems, Foster City, CA, USA) is a real time RT-PCR assay. It requires the use of the ABI Prism 7700 – a combined thermal cycler/detector (Perkin-Elmer-Applied Biosystems, Foster City, CA, USA) which utilises sequential laser stimulation of its 96 reaction wells to detect fluorescence between 500 nm and 660 nm. The fluorescence that is detected is then analysed to quantify the amplicon of interest in the samples being analysed. Presented below is a schematic diagram of the process involved.



After completion of reverse transcription the assay requires the annealing of specific oligonucleotide sequences to the cDNA under examination to be successful. The endpoints of the amplicon are defined by two specific oligonucleotide primers. This provides the first level of specificity with additional specificity from the use of an oligonucleotide probe that hybridises to the amplicon during the annealing/extension phase of the PCR. To avoid the amplification of contaminant genomic DNA the primers should be designed to span an intron. This is sometimes not possible and in such cases the RNA to be quantified should be DNAse treated with RNAse free DNAse to minimise the potential for genomic contamination of cDNA.

The assay utilises the 5'-3' nuclease activity of the DNA polymerase, Taq polymerase, to hydrolyse a hybridisation probe that is bound to its amplicon. The probe used for a Taqman assay is a 5'labelled FAM (6-carboxyfluorescene; a fluorophore) 3'labelled TAMRA (6-carboxytetramethylrhodamine; a quencher) FRET (Fluorescence Resonance Energy Transfer) probe. In FRET probes, the fluorophore donor transfers its energy to the quencher suppressing fluorescence due to its close proximity. In the absence of an amplicon complimentary to the probe in the PCR, the probe remains unbound and its fluorescence cannot be emitted for reporting. If the correct amplicon has been amplified, the probe can hybridise to that amplicon after the denaturation step. It remains hybridised while the polymerase extends the primers until it reaches the hybridised probes, when it displaces its 5' end to hold it in a forked structure. The enzyme continues to move from the now free end to the bifurcation of the duplex, where cleavage takes place. This separates the FAM reporter and TAMRA quencher dyes meaning the quencher can no longer suppress the reporter. Hence, the fluorescence is increased allowing measurement of the amount of PCR product (Bustin, 2000) (Figure 3.3).

Figure 3.3 Diagrammatic representation of Taqman real time RT-PCR process. Adapted from Taqman PCR reagent kit protocol.



3.3.4.1 Primer/Probe Design and Validation

The primers and probes used in the Taqman system need to be designed to very exacting specifications. The Taqman system incorporates its own design program, Primer express, to facilitate adherence to the design specifications for the Taqman oligonucleotides that will in turn ensure successful hybridisation with the template cDNA and efficient amplification. BLAST (Basic Local Alignment Search Tool) searches were also conducted to determine the presence of sequences in the scientific databases that are similar to those amplified by each set of primers.

Multiplexing is the use of more than one primer pair in the same tube. In the experiments, a multiplexing strategy was utilised to allow relative quantitation of cDNA between samples. In relative quantitation, one primer pair amplifies the target and the other amplifies an endogenous reference in the same tube. An endogenous reference is employed as there may be difference in the amount of starting material between samples. The accepted method for minimising these errors and correcting for sample-to-sample variation is to amplify, simultaneously with the target, a cellular RNA that serves as an internal reference against which other RNA values can be normalised (Karge *et al.*, 1998). The ideal internal standard should be expressed at a constant level among different tissues, at all stages of development, and should be unaffected by any experimental treatment. Currently three references are used; β-Actin, GAPDH and ribosomal RNA (rRNA). The endogenous reference used in these experiments was rRNA. It was used as it constitutes 85-90% of cellular RNA, their levels are unlikely to vary under conditions that may affect expression of mRNA (Barbu and Dautry, 1989) and it has also been shown to be more reliable than the other two normalisation genes in a comparison study of human malignant cell lines (Zhong and Simons, 1999).

VIC-labelled ribosomal 18s RNA probe and primers were used for normalisation in this research project (Perkin-Elmer, Applied Biosystems, Warrington, UK). VIC is an alternative fluorescent probe that is detected at a different wavelength from FAM hence allowing discrimination between the two different labelled probes. To prevent consumption of reagents by the abundant 18s within the tissue samples, the concentration of 18s primers and probes were limited to the same degree in all samples in accordance with the suggested protocol by applied biosystems.

Prior to using the primers and probes for quantitation, the linearity of the response was determined by serial dilutions of both measuring primer probe amplification against a standard pool of RNA (cDNA) that expresses the target gene. The dilutions were upto 1/64. The log of ng total RNA was plotted against the ΔCt. The Ct is the cycle number at which the PCR signal crosses a designated threshold; and the ΔCt is the difference between the Ct values for the specific amplicon and 18s. The gradient of the best fit line through these points should be < 0.1.

3.3.4.2 Taqman Protocol

Real-Time quantitative RT-PCR was used to measure the levels of PR, ERα, ERβ, AR, 3βHSD types 1 and 2 and 17βHSD types 2 and 5 in the cDNA samples. A reaction mixture containing Stratagene Taqman buffer, $MgCl_2$ (5.5mmol/l), Sure Start Taq DNA polymerase (0.025 IU/μl) (Stratagene, Amsterdam, Netherlands), dATP (200 μmol/l), dCTP (200 μmol/l), dGTP (200 μmol/l), dUTP (200 μmol/l), specific target amplicon forward and reverse probes (300 nmol/l) (Biosource, Nivelles, Belgium) and specific probe (200 nmol/l) (Biosource, Nivelles, Belgium). Additionally specific primer and probe for ribosomal 18s were also added at 50nmol/l (Perkin-Elmer; Applied Biosystems, Warrington, UK). The validated primers and probes for 3βHSD1, 3βHSD2 and 17βHSD5 were 'Assay on Demand' products supplied by PE Applied Biosystems and these primers were intron-spanning.

The mixture was then aliquoted into separate tubes for each cDNA sample. An amount of 2.5μl/replicate of 1:2.5 dilution cDNA was added to each tube. After mixing, 23μl of each sample were added to the wells on a PCR reaction plate with each sample being added and analysed in triplicate. Wells were sealed with optical caps and the PCR reaction run on the ABI prism 7700 using standard conditions.

To assess if genomic DNA contamination was present, two negative controls were added to the plate in triplicate. A no template (containing water) control and no reverse transcriptase (containing RNA) control.

3.3.5 Taqman Analysis and Statistics

The PCR data was analysed using the formula $2^{-\Delta\Delta CT}$ (as described above the Ct is the cycle number at which the PCR signal crosses a designated threshold). The

ΔCT is the difference between the Ct value at the threshold for the target amplicon and the internal normalisation control, 18S. ΔΔCT is the difference between the ΔCT and the internal reference control, which all samples are compared to, thus providing a relative value to the reference. Since each cDNA sample was analysed in triplicate the mean ΔCT was used for each.

Statistical analysis was conducted using SPSS 11.0 for Windows. Since the number of samples was small, the data were log transformed and then t-test was used to evaluate whether there were significant differences in mean target mRNA expression between samples. The error bars signify the standard error of the mean. Results with a p value of <0.05 were considered statistically significant.

CHAPTER 4

Midluteal endometrial intracrinology following controlled ovarian hyperstimulation and use of a gonadotrophin releasing hormone antagonist.

4.1 Introduction

Gonadotrophin-releasing hormone antagonists (GnRHantag), e.g. Cetrorelix and Ganirelix, are now widely used in assisted conception treatments (Albano *et al.*, 2000; Olivennes *et al.*, 2000; The European and Middle East Orgalutran Study Group, 2001; The North American Ganirelix Study Group, 2001). They cause rapid suppression of LH levels and have been found to reliably prevent premature LH surges as a part of controlled ovarian hyperstimulation (COH) in IVF/ICSI treatment (Albano *et al.*, 2000). Their use shortens the treatment cycle and also reduces the total amount of required gonadotrophins. Furthermore, they appear to reduce the incidence of ovarian hyperstimulation syndrome (Ludwig *et al.*, 2001; Al-Inany *et al.*, 2006). However, in comparison to the 'long protocol' with GnRH agonists, with the use of GnRH antagonists there is an ongoing debate regarding pregnancy rates. Whereas some studies have found similar pregnancy rates (Ludwig *et al.*, 2001), others have raised concerns about a drop in pregnancy rates (Al-Inany *et al.*, 2006).

Despite the advances in assisted conception practices, pregnancy rates are approximately 20–25%. In stimulated cycles, the endometrium is exposed to supraphysiological steroid hormone levels during the follicular phase and this might be responsible for an altered steroid receptor expression profile in the early luteal phase (Papanikolaou E, 2005). After treatment with recombinant FSH (rFSH) and a GnRH antagonist, endometrial histological advancement at the time of oocyte retrieval has been observed (Kolibianakis *et al.*, 2002). There are limited data however on the state of the endometrium during the putative window of implantation with the use of the agents. Although not clearly defined, the window of implantation is described as from Day 6 to Day 10 after the LH surge (Sharkey and Smith, 2003).

GnRH antagonists and rFSH may impact the processes of implantation through direct effects on the endometrium or indirectly through sex steroid availability and activity. Estrogens (Norwitz *et al.*, 2001; Ma *et al.*, 2003), progestogens (Lessey, 2003) and probably also androgens (Apparao *et al.*, 2002) are thought to play vital, but as yet not fully defined, roles in the complex mechanisms underlying endometrial development leading up to and after embryo implantation. These

hormones act via their cognate receptors. An alteration of the receptor expression profile could lead to changes in the function of the respective steroid hormone. Intracellular ligand availability could also influence endometrial receptivity. The enzyme 3β-hydroxysteroid dehydrogenase/Δ5- Δ4-isomerase (3βHSD) is involved in the biosynthesis of all classes of active steroids. Pregnenolone is converted to progesterone under the effect of 3βHSD in the human endometrium and this might be crucial for implantation and maintenance of pregnancy. In the secretory phase, 3βHSD is moderately expressed in the glandular epithelium of the endometrium (Rhee *et al.*, 2003). The enzymes 17βHSD2 and 17βHSD5 have been identified in the human endometrium. The 17βHSD5 transforms not only androstenedione to testosterone and estrone to estradiol (E2), but also progesterone to 20-hydroxyprogesterone. In the endometrium, its expression has been localized to the surface epithelium and the vascular endothelium (Pelletier *et al.*, 1999). The 17βHSD2 has a major role in the inactivation of E2 to estrone. It is also responsible for converting androgens to less potent forms, while also activating progesterone. It is expressed in endometrial glandular epithelium, and is up-regulated by progesterone (Maentausta *et al.*, 1993). The availability of various androgenic ligands to bind to the androgen receptor (AR) may be influenced by the local presence of 17βHSD2 (Burton *et al.*, 2003). The activity of 17βHSD2 has been localized predominantly in the glandular epithelium but also in the endometrial stroma. It has been postulated that the anti-estrogen action of progesterone in the endometrial glands is mediated through this enzyme (Casey *et al.*, 1994; Burton *et al.*, 2003).

The aim of this study was to compare the untreated mid-luteal endometrium with the endometrium during the putative window of implantation after treatment with rFSH and GnRH antagonists, by mimicking the exact conditions that would be expected to occur in an IVF/ICSI treatment cycle. It was also to determine the endometrial intracrinology in relation to expression of sex-steroid receptors and steroid metabolizing enzymes during the putative window of implantation.

4.2 Methods

First, the sex steroid receptor expression during the mid-secretory phase was studied. Sex steroid receptor protein expression was studied with immunohistochemistry (IHC) and the mRNA expression was studied with real time quantitative – polymerase chain reaction (QRT-PCR). Second, the steroid metabolising enzymes expression during the mid-secretory phase was evaluated. Enzyme expression was studied with IHC and mRNA expression was studied with QRT-PCR.

All endometrial samples were histologically classified as per the Noyes criteria (Noyes *et al.*, 1950) to confirm the mid-luteal stage of the cycle.

4.2.1 Patient characteristics:

Institutional ethical approval was obtained and all women gave informed written consent. The study group consisted of parous women who had volunteered to donate oocytes and women in the control group consisted of healthy parous women with regular menses (25-35 days). The sample sizes and age demographics of the women taking part in the respective parts of the study are presented in Tables 4.1(a) – (d). The women included in the study group, i.e. the oocyte donors, underwent a cycle of ovarian stimulation as per the Edinburgh Assisted Conception Unit's protocol (Thong *et al.*, 2003). Recombinant-FSH (Gonal-F; Serono, UK) was commenced on day 4 of the menstrual cycle. All donors commenced ovarian stimulation at the dose of 150IU. Gonadotrophin releasing hormone antagonist, Cetrorelix, was commenced at a dose of 0.25mg daily on day 7 or 8 of their cycle once at least 2 or more follicles had reached the size of 11mm diameter. Ovarian response was monitored by transvaginal ultrasound from day 4. When the three largest follicles measured $\geq$ 17mm, oocyte maturation was triggered by the administration of a single subcutaneous injection of 10000U hCG (Profasi; Serono, UK). Oocyte retrieval was performed 35-36 hours after hCG administration. Progesterone (Cyclogest; Alpharma, UK) vaginal pessaries (200mg.) were administered 12 hourly starting 2 days after oocyte retrieval and up to the day of endometrial sampling. A pipelle endometrial biopsy (EB) was conducted 8-10 days after hCG administration (Table 4.2(a)).

Table 4.1 Age demographics of participants in respective parts of the studies

(a) – Sex steroid receptor protein expression

Immunohistochemistry study			
	Control	Study	P value
Subject numbers*(n)*	*8*	*5*	
Mean age (years)	38.0 ± 2.7	31.0 ± 4.3	0.66

(b) – Sex steroid receptor quantitative mRNA expression

QRT-PCR study			
	Control	Study	P value
Subject numbers*(n)*	*5*	*4*	
Mean age (years)	41.4 ± 2.4	31.0 ± 4.9	0.92

(c) - Steroid metabolizing enzyme protein expression

Immunohistochemistry study			
	Control	Study	P value
Subject numbers*(n)*	*6*	*5*	
Mean age (years)	35.6 ± 3.7	31.0 ± 4.3	0.92

(d) – Steroid metabolizing enzyme quantitative mRNA expression

QRT-PCR study			
	Control	Study	P value
Subject numbers*(n)*	*4*	*3*	
Mean age (years)	41.7 ± 2.6	30.0 ± 5.5	0.97

Table 4.2 Biopsy timing in relation to hCG injection (study group) or urinary LH surge (control group)

(a) Study group (n=5)

Participant Number	Biopsy day (from HCG)
1	9
2	8
3	10
4	8
5	10

(b) Control Group (n=8)

Participant Number	Biopsy day (after LH surge)	Progesterone level (nmol/l)
1	8	28.4
2	9	39.3
3	7	40.8
4	7	37
5	8	33.6
6	8	35
7	9	55.1
8	7	78.1

Note: The study design did not include taking blood samples for progesterone levels from participants in the Study group. Women in the study group were oocyte donors who were undergoing a cycle of ovarian stimulation and since they underwent an oocyte retrieval, their 'ovulatory' status was not in doubt. While it is recognised that this information would have been useful, it would have been difficult to draw conclusions from progesterone levels when one group was more likely to have uni-follicular ovulation (Control group) and the other group would have had multi-follicular 'ovulation' (Study group).

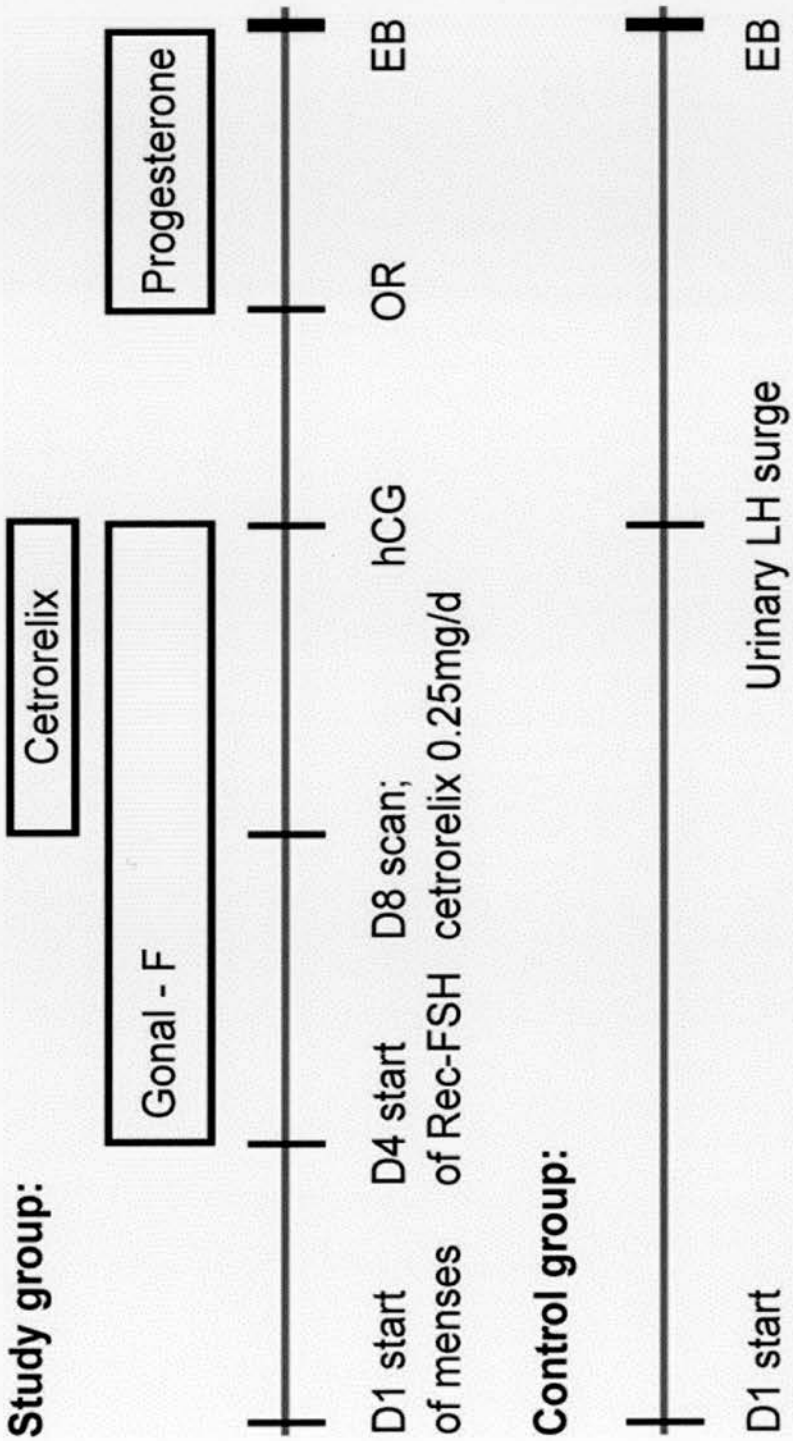
The control group consisted of healthy parous ovulatory women with regular menses (25-35 days) (Tables 4.1 (a) – (d)). Only parous women who attended the gynaecology clinics with requests for sterilization or other complaints excluding menstrual problems or infertility were included in the control group. Endometrial biopsies from 8 of these women were available for the IHC studies. To help time the endometrial biopsies, these women were asked to provide a urine sample on alternate days from day 10 of their last menstrual period. Urinary LH levels were

measured. An EB was performed 6-10 days (Table 4.2(b)) after the peak of a urinary LH surge i.e. in the mid-luteal stage of the cycle (Figure 4.1). For confirmation of ovulation a blood sample was obtained on the day of their endometrial biopsy and the progesterone values observed are presented in Table 4.2 (b).

In a urine sample, an LH surge would be detected a day after it has actually occurred. Hence to ensure all suitable control women could be included in the study we have 2 extra days allowed in the protocol for control subjects but this would still ensure that the biopsies were within the window of implantation. Whereas most of the endometrial biopsies were conducted in the middle of the window of implantation (confirmed on histological dating), the extra days were allowed in the protocol so that as many women as possible could be included. The accuracy of endometrial dating was confirmed not only by serum progesterone levels but also by histological examination of the endometrium.

There are limited data relating to sex steroid receptor expression in mid-luteal phase endometrium. The one study by Simon and colleagues did not find any significant changes in PR and ER expression at LH+7 after treatment with Ganirelix when compared to mid-luteal (LH+7) untreated controls (also referred to above). The same study did not find any difference in gene expression between natural cycle and Ganirelix treated endometrium at LH+7 (Simon C *et al.*, 2005).

Figure 4.1 Diagram depicting the timing of endometrial biopsy in the study and control groups. OR – Oocyte Recovery, EB – Endometrial Biopsy



Endometrial tissue from a subset of five parous women was available for QRT–PCR studies. In these women, the LMP, endometrial histology and serum progesterone levels were all consistent with mid-luteal phase of the cycle. This subset was chosen on the basis of mRNA quality. Although the mean age difference between women in the two groups was substantial, since the aim of this part of the study was quantitative analysis rather than qualitative analysis, it was decided to include these women in this part of the study. To my knowledge, there are no data to suggest that there are any quantitative mRNA changes in the endometrium of women at different ages. Hence, accepting the limitations of interpreting results in the background of a substantial difference in ages of these women, in the absence of evidence to the contrary, for the current study, it was assumed that as long as it was ensured that the endometrium was in the mid-luteal stage, the quantitative data should be valid.

Endometrial tissue was fixed in 4% paraformaldehyde then embedded in paraffin for immunohistochemical analysis. In addition, endometrium was also frozen at the point of tissue collection in liquid nitrogen and stored at -70°C. RNA was extracted from frozen endometrial tissue.

4.2.2 Immunohistochemistry

The expression of PR, ERα, ERβ and AR and enzyme expression of 3βHSD and 17βHSD5 were studied with immunohistochemistry. The 3βHSD antibody recognizes both human 3βHSD1 and 3βHSD2 enzymes. No documented antibodies were available for 17βHSD2 enzyme and hence no immunochemistry was performed for this enzyme.

The general immunohistochemistry protocol followed is described in section 3.2.1. Table 4.3 summarises the incubation conditions for immunolocalisation of each of the epitopes studied. Commercially available antibodies were used for immunolocalisation of endometrial PR, ERα, ERβ and AR. The 3βHSD rabbit polyclonal antibody was raised against recombinant human 3βHSD2 and with similar affinity recognizes both human 3βHSD1 and 3βHSD2 (McDonald SE, Mason JI, Critchley HOD, unpublished observations). A mouse monoclonal antibody against human 17HSD5 (Lin *et al.*, 2004) was a generous gift from Dr

Trevor Penning (University of Pennsylvania, Philadelphia, USA). Neither a mouse monoclonal antibody against human 17βHSD2 which was used in an earlier study (Burton *et al.*, 2003), nor any commercial 17HSD2 antibody were currently available and hence no immunochemistry was performed for this enzyme.

Table 4.3 Summary of incubation conditions for Immunohistochemistry

Protein of interest	**Antigen Retrieval Pressurecook(PC) Microwave(MW)**	**Primary Antibody**	**Negative control**
Progesterone receptor (PR)	MW Buffer – 0.01M Na Citrate	Monoclonal mouse anti PR antibody, Novocastra, Newcastle, UK 1:40 in normal horse serum	Mouse Immuno-globulin IgG, Sigma, Dorset,UK 1:800
Estrogen receptor α (ERα)	MW Buffer – 0.01M Na Citrate	Monoclonal mouse anti ERα antibody, Dako,Cambridge UK 1:400 in PBS	Mouse Immuno-globulin IgG, Sigma, Dorset,UK 1:2400 in PBS
Estrogen receptor β (ERβ)	PC Buffer – 0.05M Glycine/0.01% EDTA (pH 8)	Monoclonal mouse anti-ERβ antibody, Serotec, Oxford, UK in NRS/TBS/BSA	NRS/TBS/BSA
Androgen receptor (AR)	PC Buffer – 0.01M Na Citrate	Monoclonal mouse anti AR antibody, Biogenex,CA USA 1:240 Overnight incubation at 4°C	Mouse Immuno-globulin IgG, Sigma, Dorset,UK 1:300
3β-hydroxysteroid dehydrogenase (3βHSD)	No antigen retrieval performed	Polyclonal rabbit anti-3βHSD (recognises both isoforms), 1:500 in NGS/PBS/BSA	Pre-immune serum, 1:500 in NGS/PBS/BSA
17β-hydroxysteroid dehydrogenase type 5 (17βHSD5)	PC Buffer – 0.01M NaCitrate (pH6)	Monoclonal mouse anti-17βHSD-5, 1:200 in NHS/PBS/BSA (Lin *et al.*, 2004)	Mouse Immunoglobulin G, Sigma, Dorset UK, 1:300 in NHS/PBS/BSA

4.2.3 RNA extraction and RT-PCR

Frozen samples of endometrium stored at -70°C were homogenized and then total RNA was extracted using Trizol (Invitrogen Life Technologies Ltd, UK) according to the manufacturer's instructions. The genomic DNA was removed by subjecting the RNA to DNAse treatment. After extraction the concentration and quality of RNA were assessed using an Agilent bioanalyzer (Agilent Technologies, South Queensferry, West Lothian, UK). The RT-PCR reaction was performed as described before (Section 3.3; Henderson *et al.*, 2003; McDonald *et al.*, 2006). In brief, a 10 µl volume of reaction solution containing the following: 1×Taqman RT buffer, magnesium chloride, deoxyNTPs, random hexamers, Multiscribe reverse transcriptase, RNase inhibitor and nuclease-free water (reagents from Applied Biosystems, Cheshire, UK) was used. 200 ng of template RNA was added. The RT reaction was conducted at 25 °C for 60 min, 48 °C for 45 min and 95 °C for 5 min for one cycle. RT-negative control had template RNA but no multiscribe enzyme included, and an RT H_2O had template RNA replaced by nuclease free water. Negative controls were included in every run. The samples were then stored at −20 °C.

4.2.4 Quantitative real-time PCR (QRT–PCR)

The primer/probe sets were designed using the Primer Express program (PE Applied Biosystems) as described before (Section 3.3; Henderson *et al.*, 2003) or purchased from PE Applied Biosystems' Assay on Demand service. Where possible these were chosen to span an intron to further reduce the chance of spurious readings due to genomic DNA contamination. The sequences of the primer/probe sets and their location within the specified cDNAs are given in Table 4.4. The 18S primers and probe were purchased from PE Applied Biosystems. A Taqman real-time PCR mix was then prepared containing final concentrations of Taqman universal PCR master mix (1x), ribosomal 18S forward and reverse primers, and probe (50 nM; PE Applied Biosystems), forward and reverse primers (300 nM), and probe for sequence of interest (200 nM; PE Applied Biosystems). Wells were sealed with optical caps and the PCR was run on the Perkin-Elmer ABI Prism 7900 (PE Applied Biosystems) using standard conditions.

The PR, ERα, ERβ and AR mRNA and 3βHSD types 1 and 2 and 17βHSD types 2 and 5 mRNA levels were studied with QRT-PCR. Taqman QRT-PCR was carried out with primers and probes specific for the PR, ERα, ERβ, AR, 3βHSD types 1 and 2 and 17βHSD types 2 and 5. The validated primers and probes for 3βHSD1, 3βHSD2 and 17βHSD5 were 'Assay on Demand' products supplied by PE Applied Biosystems and these primers were intron-spanning.

Table 4.4 Steroid receptor primer and probe sequences used for amplification by real time QRT-PCR

Primer/probe	Sequence	Position	Accession no.
ERβ1 forward	CCTGGCTAACCTCCTGATGCT	1459–1480	AB006590
ERβ1 reverse	CCACATTTTTGCACTTCATGTTG	1529–1552 (r)	AB006590
ERβ1 probe	AGATGTTCCATGCCCTTGTTACTCGCA	1499–1525 (r)	AB006590
ERα forward	TGATTGGTCTCGTCTGGCG	1523–1541	NM_000125
ERα reverse	CATGCCCTCTACACATTTTCCC	1602–1624 (r)	NM_000125
ERα probe	TGCTCCTAACTTGCTCTTGGACAGGAACC	1572–1600	NM_000125
PR forward	CAGTGGGCGTTCCAAATGA	2151–2170	NM_000926
PR reverse	TGGTGGAATCAACTGTATGTCTTGA	2209–2233 (r)	NM_000926
PR probe	AGCCAAGCCCTAAGCCAGAGATTCACTT T	2170–2199	NM_000926
AR forward	GTACCCTGGCGGCATGGT	951–1016	L29496
AR reverse	CCCATTTCGCTTTTGACACA	951–1016	L29496
AR probe	AGCAGAGTGCCCTATCCCAGTCCCA	951–1016	L29496
17βHSD-2 forward	TGTCAGCAGCATGGGAGGA	731–803	L11708
17βHSD-2 reverse	GGTCACAGCCGCCTTTGAT	731–803	L11708
17βHSD-2 probe	CCCCAATGGAAAGGCTGGCATCTT	731–803	L11708
3βHSD1	Assay on demand		Hs00426435_m1
3βHSD2	Assay on demand		Hs00605123_m1
17βHSD5(AKR1C3)	Assay on demand		Hs00366267_m1

The positions of the sequences are given within the cDNA, identified by the accession number; r denotes reverse strand.

4.2.5 Scoring, data presentation and statistical analysis of immunoreactivity.

The immunostaining intensity of epitopes in all tissue sections was assessed in a semi-quantitative manner on a 4 point scale: 0 = no staining; 1 = mild/minimal immunostaining; 2 = moderate immunostaining; 3 = intense immunostaining. All tissue sections were scored blind by at least 2 observers. The semi-quantitative immunohistochemistry data were analysed using the Mann-Whitney test. The QRT-PCR data were log transformed and then the t-test was used to test for statistical significance.

4.3 Results

4.3.1 Endometrial histology

In patients treated with a rFSH–GnRH antagonist, almost all biopsies with adequate tissue showed histological features consistent with mid-secretory phase of the cycle. In one biopsy, there were features suggestive of advancement of the dates and this was reported to be consistent with early to mid-secretory phase endometrium. One endometrial biopsy sample submitted for histology was unsatisfactory and hence histology on that sample could not be analysed (Table 4.5).

Immunoexpression of PR, ERα, ERβ, AR, 3βHSD and 17βHSD5 at five cellular locations, i.e. endometrial glands, stroma, surface epithelium, vascular endothelium and perivascular cells, was studied. The mRNA transcripts of PR, ERα, ERβ, AR, 3βHSD1, 3βHSD2, 17βHSD2 and 17βHSD5 were evaluated.

Table 4.5 Histological analysis (dating) of endometrial biopsies of women treated with a rFSH–GnRH antagonist

Biopsy number	Histology report
1	Mid-secretory phase endometrium
2	Mid-secretory phase endometrium
3	Mid-secretory phase endometrium
4	Early to Mid-secretory phase endometrium
5	Unsatisfactory specimen

4.3.2 Immunohistochemistry

Sex-steroid receptors

PR immunoexpression (Table 4.6, Figures 4.2 and 4.3)

Compared with endometrium from untreated women, endometrium exposed to Gonal-F® and Cetrorelix showed significantly reduced immunoexpression in stroma ($P < 0.05$) and surface epithelium ($P < 0.05$). Conversely, PR immunoreactivity was significantly increased in the perivascular cells ($P < 0.05$). The difference in PR immunoexpression in the glands was significant ($P < 0.05$), but we observed a marked variability in expression among the biopsies in the control group.

ERα immunoexpression (Table 4.6, Figures 4.2 and 4.3)

Mild immunoexpression of ERα was observed at most cellular locations. No ERα immunoexpression was observed in the vascular endothelium. No significant differences were observed in endometrial ERα immunostaining between the two groups of women.

ERβ immunoexpression (Table 4.6, Figures 4.2 and 4.3)

ERβ immunoexpression at most cellular locations was consistently strong with or without treatment with GnRH antagonist and rFSH. Stromal expression was less intense, but overall no significant differences were observed in endometrial ERβ immunoexpression between the two groups of subjects.

AR immunoexpression (Table 4.6, Figure 4.2)

AR immunoexpression was negligible in the glandular epithelium with or without COH treatment. Stromal immunoexpression of AR was moderate, but overall no significant differences were observed in endometrial AR immunostaining between the two groups of subjects.

Table 4.6 Immunohistochemical semi-quantitative scoring results – Steroid receptor expression data

Progesterone Receptor expression			
Cellular compartment	Control (n=8) Mean scores ± SD	Study (n=5) Mean scores ± SD	*p* value
Glands	1.88 ± 0.83	0 ± 0	0.003*
Stroma	3 ± 0	2.2 ± 0.45	0.019*
Surface Epithelium	2.25 ± 0.89	0.8 ± 0.45	0.019*
Vascular Endothelium	No immuno-reactivity seen	No immuno-reactivity seen	
Perivascular cells	0.38 ± 0.52	1.4 ± 0.55	0.023*

*$p < 0.05$ denotes statistical significance (Mann-Whitney test)

Estrogen Receptor α expression			
Cellular compartment	Control (n=8) Mean scores ± SD	Study (n=5) Mean scores ± SD	*p* value
Glands	1.13 ± 0.35	0.4 ± 0.55	0.057
Stroma	1.63 ± 0.74	1 ± 0.71	0.213
Surface Epithelium	1.5 ± 0.53	1.8 ± 0.84	0.558
Vascular Endothelium	No immuno-reactivity seen	No immuno-reactivity seen	
Perivascular cells	1.5 ± 0.53	1.6 ± 0.55	0.769

$p < 0.05$ denotes statistical significance (Mann-Whitney test)

Estrogen Receptor β expression			
Cellular compartment	Control (n=8) Mean scores ± SD	Study (n=5) Mean scores ± SD	*p* value
Glands	2.38 ± 0.52	2.2 ± 0.45	0.523
Stroma	1.25 ± 0.46	1.8 ± 0.45	0.063
Surface Epithelium	2 ± 0.93	1.8 ± 0.45	0.694
Vascular Endothelium	1.88 ± 0.35	1.6 ± 0.55	0.271
Perivascular cells	1.63 ± 0.52	1.4 ± 0.55	0.446

$p < 0.05$ denotes statistical significance (Mann-Whitney test)

Androgen Receptor expression			
Cellular compartment	Control (n=8) Mean scores ± SD	Study (n=5) Mean scores ± SD	*p* value
Glands	0.5 ± 0.53	0.2 ± 0.45	0.379
Stroma	2 ± 0.76	1.6 ± 0.55	0.379
Surface Epithelium	0.13 ± 0.35	0.2 ± 0.45	0.826
Vascular Endothelium	0.25 ± 0.46	0 ± 0	0.464
Perivascular cells	0.75 ± 0.71	1 ± 0	0.464

$p < 0.05$ denotes statistical significance (Mann-Whitney test)

Figure 4.2 Immunoreactivity scores in different endometrial cellular compartments. Note decreased PR in endometrial stroma (P=0.019)*, surface epithelium (P=0.019)* and glands (P=0.003)* and increased PR in perivascular cells (P=0.023)*. Box-and-whisker plots: box represents the 25th and 75th percentiles, whiskers represent the 10th and 90th percentiles and the heavy bar represents the median. □ = Control, ■ = Study

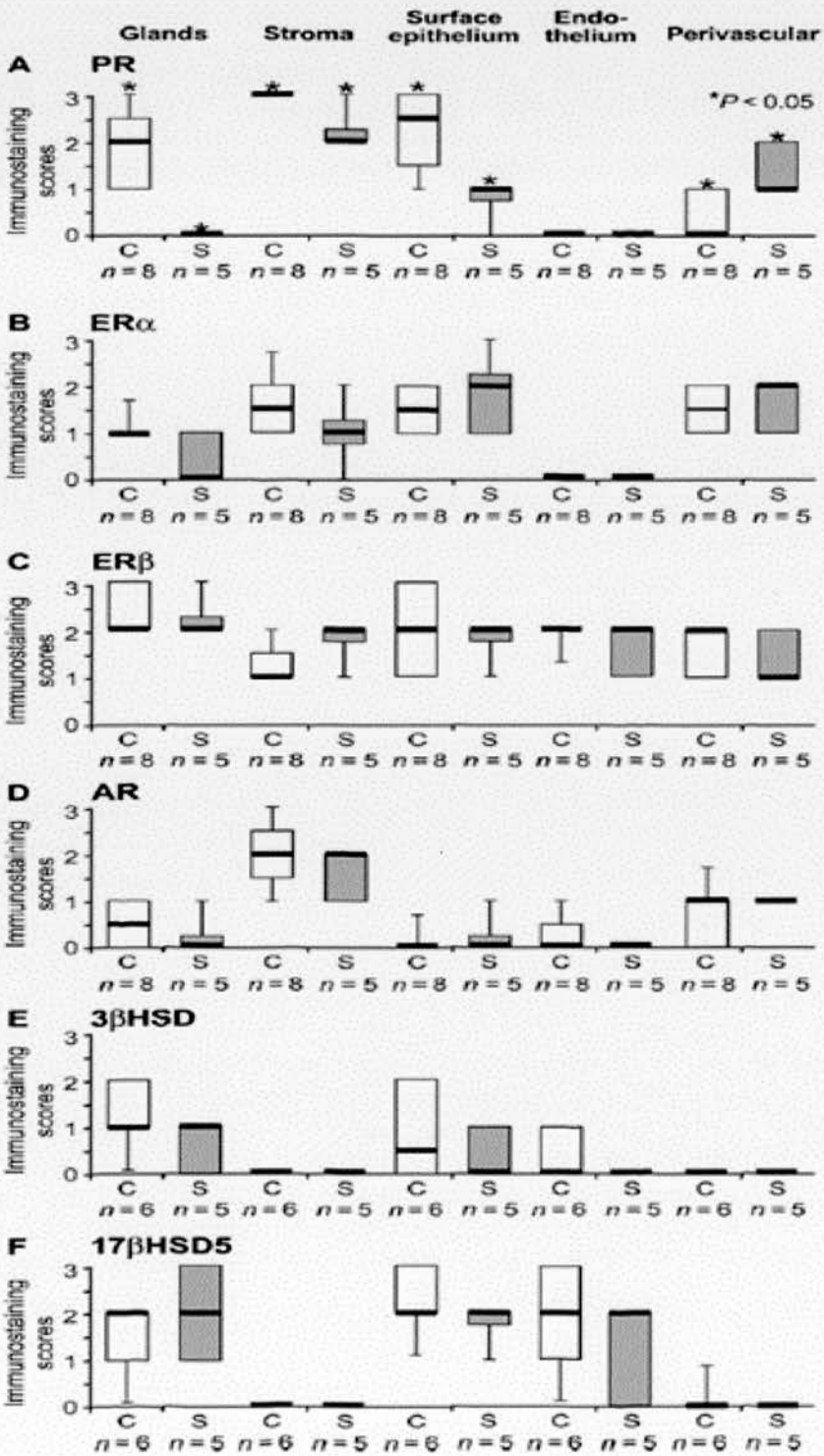
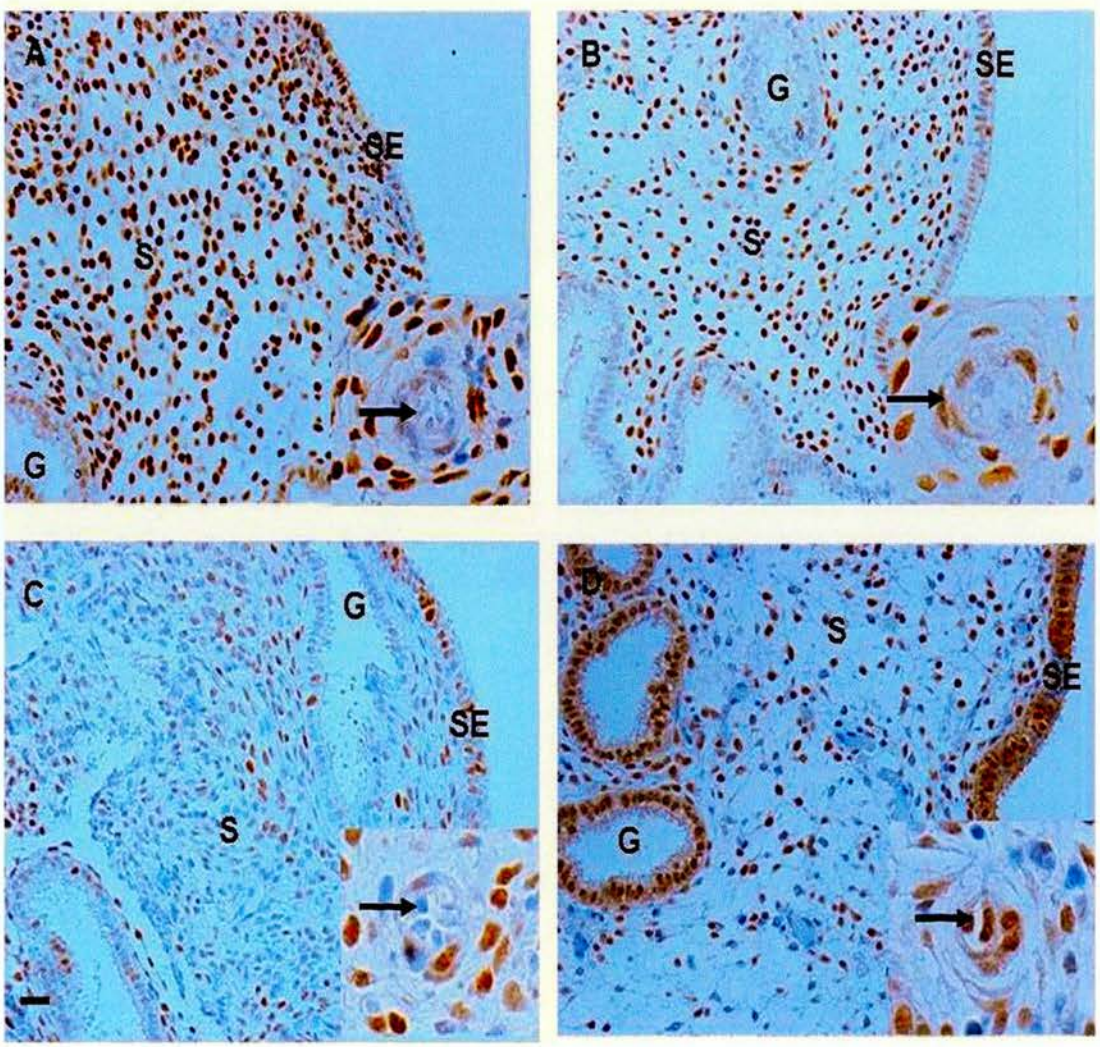


Figure 4.3 Immunohistochemical localisation of steroid receptors in human endometrium of women in the mid-luteal phase of cycle (control group) and in women who have received COH and a GnRHantag (study group). (A) PR immunostaining in endometrium from untreated women in mid-luteal phase – intense stromal(S) and surface epithelial (SE) immunoreactivity (A) Inset - note low level of perivascular immunostaining (arrow). (B) PR immunostaining in the endometrium from women treated with GnRHantag and rFSH – reduced stromal and surface epithelial immunostaining. (B) Inset - more intense perivascular cell immunostaining (arrow). (C) ERα immunostaining in endometrium from women treated with rFSH and GnRHantag – mild immunostaining at all cellular locations. (C) Inset – absent immunostaining in the vascular endothelium (arrow). (D) ERβ immunostaining in endometrium from women treated with rFSH and GnRHantag. – strong immunoreactivity in all cell types. (D) Inset – strong ERβ immunostaining in vascular endothelium. Scale Bar = 20 microns. G = Glands, S = Stroma, SE = Surface Epithelium



Steroid metabolizing enzymes

3βHSD immunoexpression (Table 4.7, Figures 4.2 and 4.4)

Irrespective of COH, expression of 3βHSD in the glands, surface epithelium and endothelium was at a low level. No immunoexpression was seen in stroma or in perivascular cells. No significant differences were observed in endometrial 3βHSD immunostaining between the two groups of subjects.

17βHSD5 immunoexpression (Table 4.7, Figures 4.2 and 4.4)

Moderate to intense 17βHSD5 immunoexpression was observed in the endometrial glands, and surface epithelium with or without treatment. No immunoexpression was observed in stroma, however, negligible immunoexpression was observed in perivascular cells. Moderate immunoexpression was observed in the vascular endothelium. No significant differences were observed in endometrial 17βHSD5 immunostaining between the two groups of subjects.

Table 4.7 Immunohistochemical semi-quantitative scoring results – Steroid metabolizing enzyme expression data

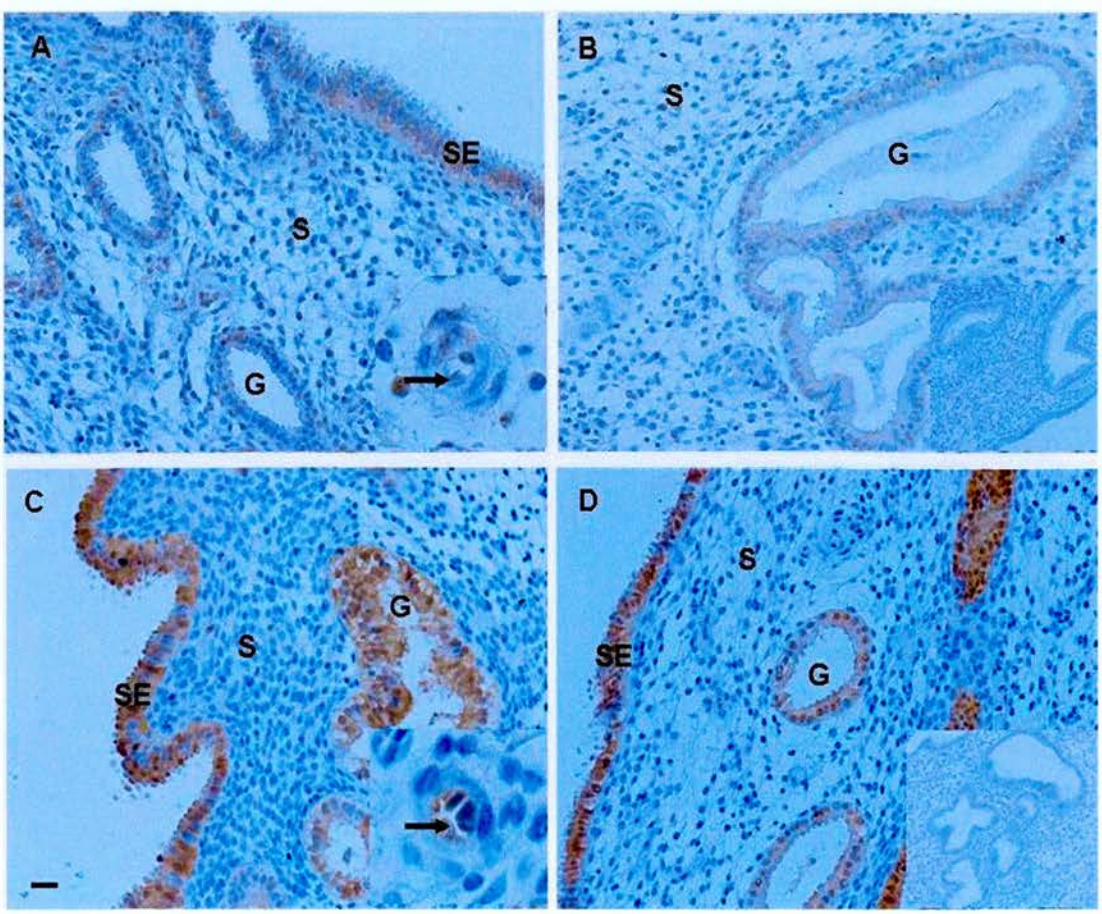
3β Hydroxysteroid Dehydrogenase expression			
Cellular compartment	Control (n=6) Mean scores ± SD	Study (n=5) Mean scores ± SD	*p* value
Glands	1.17 ± 0.75	0.6 ± 0.55	0.235
Stroma	No immuno-reactivity seen	No immuno-reactivity seen	
Surface Epithelium	0.83 ± 0.98	0.4 ± 0.55	0.522
Vascular Endothelium	0.33 ± 0.52	0 ± 0	0.361
Perivascular cells	No immuno-reactivity seen	No immuno-reactivity seen	

$p < 0.05$ denotes statistical significance (Mann-Whitney test)

17β Hydroxysteroid Dehydrogenase type 5 expression			
Cellular compartment	Control (n=6) Mean scores ± SD	Study (n=5) Mean scores ± SD	*p* value
Glands	1.5 ± 0.84	2 ± 1	0.465
Stroma	No immuno-reactivity seen	No immuno-reactivity seen	
Surface Epithelium	2.17 ± 0.75	1.8 ± 0.45	0.411
Vascular Endothelium	0.67 ± 1.03	0.4 ± 0.55	0.855
Perivascular cells	Negligible immuno-reactivity seen	No immuno-reactivity seen	

$p < 0.05$ denotes statistical significance (Mann-Whitney test)

Figure 4.4 Immunohistochemical localisation of selected steroid metabolising enzymes in human endometrium of women in the mid-luteal phase of cycle (control group) and in women who have received COH and a GnRHantag (study group). (A) 3βHSD immunostaining in endometrium from untreated women in mid-secretory phase – low level immunostaining at most cellular locations. (A) Inset: low level immunostaining in vascular endothelium (arrow). (B) 3βHSD immunostaining in the endometrium from women treated with rFSH and GnRHantag. - low level immunostaining at most cellular locations. (B) Inset: 3βHSD negative – no immunostaining (C) 17βHSD5 immunostaining in endometrium from untreated women in mid-secretory phase – moderate immunostaining in glands (G) and surface epithelium (SE). No immunostaining in stroma (S). (C) Inset: Moderate immunostaining in vascular endothelium (arrow). (D) 17βHSD5 immunostaining in endometrium from women treated with rFSH and GnRHantag. – moderate immunostaining in glands and surface epithelium. No immunostaining in stroma. (D) Inset: 17βHSD5 negative – no immunostaining. Scale Bar = 20 microns.
G = Glands, S = Stroma, SE = Surface Epithelium



4.3.3 Quantitative real-time PCR (Table 4.8, Figure 4.5)

Sex-steroid receptors

QRT–PCR demonstrated a statistically significant reduction in the amount of endometrial ERα mRNA ($P = 0.02$) and AR mRNA ($P = 0.01$) in COH treated women compared with the controls. Although the level of PR mRNA was reduced in the COH group, the difference was not significant ($P = 0.12$). No significant differences were observed in the amount of ERβ mRNA ($P = 0.96$) between the two groups.

Steroid metabolizing enzymes

The 3βHSD1 mRNA ($P = 0.01$) and 17βHSD2 mRNA ($P = 0.02$) levels were significantly reduced in the COH group. In relation to 3βHSD2 and 17βHSD5 QRT–PCR, sufficient RNA was only available in 1 and 2 study samples, respectively. Hence in these cases, the sample size was too small for analysis. However, expression of adrenal/gonadal-specific 3βHSD2 transcripts is predicted to be minimal in endometrium and the relative 3βHSD1/3βHSD2 transcript ratio observed in the one sample is supportive of a minimal 3βHSD2 contribution to endometrial 3βHSD.

Table 4.8 Sex steroid receptors and steroid metabolizing enzymes quantitative mRNA expression results

Sex steroid receptor mRNA expression			
	Control (n=5) Mean $2^{-\Delta\Delta CT}$ values ± SD	Study (n=4) Mean $2^{-\Delta\Delta CT}$ ± SD	*p* value
PR	1.39 ± 0.74	0.55 ± 0.14	0.12
ERα	1.44 ± 0.94	0.34 ± 0.14	0.02*
ERβ	0.35 ± 0.37	0.18 ± 0.17	0.96
AR	0.72 ± 0.35	0.16 ± 0.05	0.01*

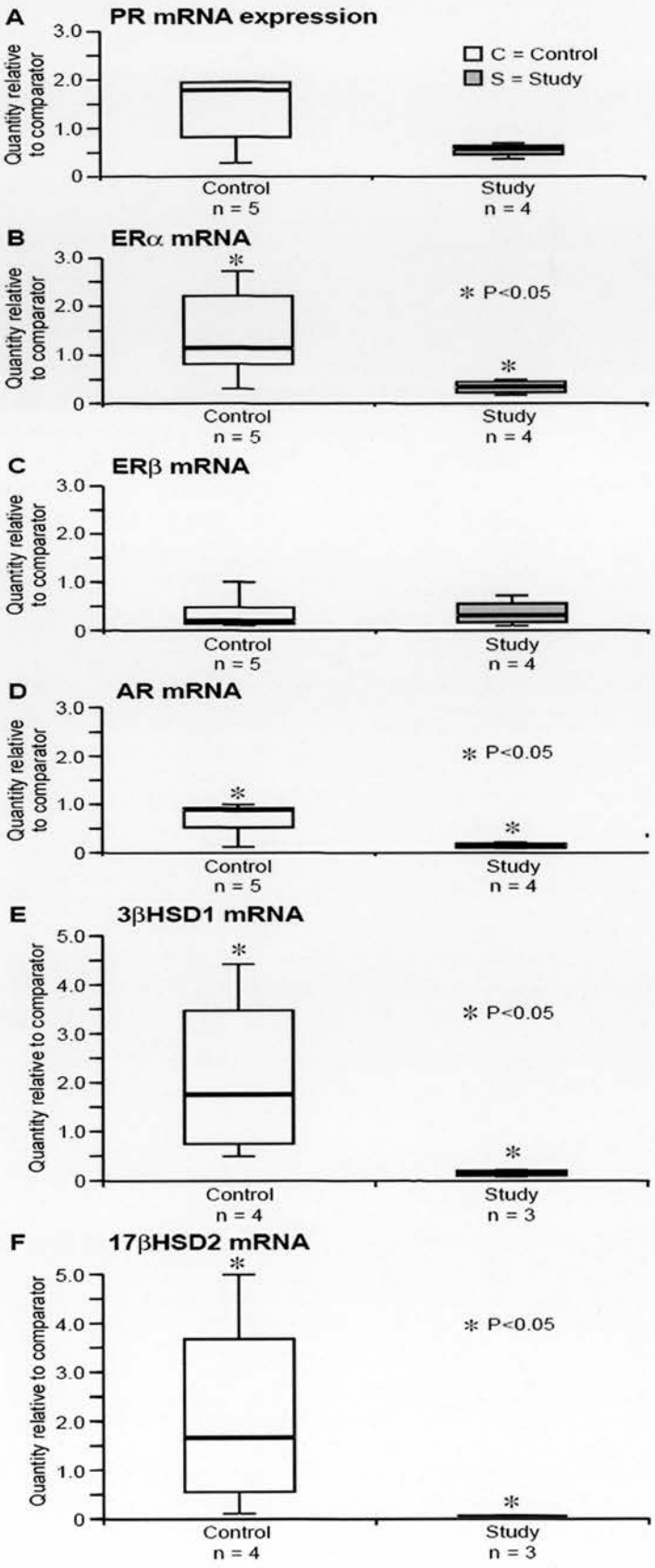
*$p < 0.05$ denotes statistical significance (t-test after log transformation)

Steroid metabolizing enzyme mRNA expression			
	Control (n=4) Mean $2^{-\Delta\Delta CT}$ values ± SD	Study (n=3) Mean $2^{-\Delta\Delta CT}$ values ± SD	*p* value
3βHSD1	2.11 ± 1.76	0.16 ± 0.07	0.012*
17βHSD2	2.13 ± 2.15	0.06 ± 0	0.028*
3βHSD2	Sample size too small for analysis		
17βHSD5	Sample size too small for analysis		

*$p < 0.05$ denotes statistical significance (t-test after log transformation)

Figure 4.5 Quantitative evaluations of sex-steroid receptors and steroid receptor metabolising enzymes mRNA expression of women in the mid-luteal phase of cycle (control group) and in women who have received COH and a GnRHantag (study group). (A) PR (PR), (B) ERα (ERα), (C) ERβ (ERβ), (D) AR (AR), (E) 3βHSD1 enzyme and (F) 17βHSD2 enzyme. All endometrial tissue samples were compared with an internal control (comparator) obtained during the mid-luteal phase of the menstrual cycle. ERα mRNA (P=0.02) and AR mRNA (P=0.01) levels were significantly reduced in endometrial tissue from women treated with GnRHantag and rFSH. 3βHSD1 mRNA (P=0.01) and 17βHSD2 mRNA (P=0.02) levels were significantly reduced in the endometrial samples from women treated with GnRHantag and rFSH. Note scale (Y axis) in E and F differs from A-D

☐ = Control, ▒ = Study


A
PR mRNA expression
C = Control
S = Study
Quantity relative to comparator
3.0
2.0
1.0
0
Control
n = 5
Study
n = 4
B
ERα mRNA
* P<0.05
Quantity relative to comparator
3.0
2.0
1.0
0
Control
n = 5
Study
n = 4
C
ERβ mRNA
Quantity relative to comparator
3.0
2.0
1.0
0
Control
n = 5
Study
n = 4
D
AR mRNA
* P<0.05
Quantity relative to comparator
3.0
2.0
1.0
0
Control
n = 5
Study
n = 4
E
3βHSD1 mRNA
* P<0.05
Quantity relative to comparator
5.0
4.0
3.0
2.0
1.0
0
Control
n = 4
Study
n = 3
F
17βHSD2 mRNA
* P<0.05
Quantity relative to comparator
5.0
4.0
3.0
2.0
1.0
0
Control
n = 4
Study
n = 3

4.4 Discussion

The use of rFSH and a GnRH antagonist is an effective and reliable regime for controlled ovarian stimulation as a part of IVF/ICSI treatment cycles. However, its effects on the endometrium especially in relation to embryo implantation have not been evaluated in detail. Although histological advancement of the chronological stage of the endometrium has been observed at the time of oocyte retrieval (Kolibianakis *et al.*, 2002), the endometrial sex-steroid receptor protein and mRNA expression levels during the putative window of implantation with the use of these agents have not been reported. Furthermore no reports have been identified describing expression of steroid metabolizing enzymes and their mRNA transcripts during the window of implantation in the mid-luteal phase of the cycle with this treatment regimen.

These studies have described a significant difference in PR protein expression in women treated with rFSH and GnRH antagonist. Significant down-regulation of PR protein expression in the endometrial stroma and surface epithelium was observed. A significant up-regulation was observed in the perivascular cells in women treated with cetrorelix and Gonal-F®. PR immunoexpression in the endometrial glands was reduced in the treatment group, although the expression in the controls was variable. No significant differences were observed in the protein expression of ERα, ERβ, AR, 3βHSD or 17βHSD5. Quantitatively, there was a significant reduction in the levels of ERα mRNA, AR mRNA, 3βHSD1 mRNA and 17βHSD2 mRNA in the treatment group. No significant difference was observed in the amount of ERβ mRNA transcripts between the two groups of women, and although PR mRNA was reduced in the treatment group, the difference was also not significant.

Healthy fertile women were recruited as the control group. Women undergoing assisted conception are a different group with alterations in physiological steroid levels and possibly in the endometrium. Hence, recruiting untreated parous women as a control group may not be the ideal equivalent comparison. However, the aim was to try and understand the differences between the physiological state of embryo implantation, which occurs in the mid-luteal phase in a natural ovulatory cycle, and in rFSH and a GnRH antagonist treated endometrium. Hence, it was

appropriate to include untreated fertile ovulatory women in the control group. In the subjects in the control group, an LH surge was detected using an alternate day urinary LH protocol. Urinary LH measurement may not be the most accurate way of checking the timing of ovulation. However, it is possible to identify the window of implantation on the basis of an alternate day urinary LH dating protocol. First, the window of implantation is thought to extend over Days 5–10 after the LH surge. Hence, even with urinary LH dating, it is possible to identify the putative window of implantation. Second, it is theoretically possible that the difference of 1 or 2 days could influence the result. The endometrium has been dated not only by LH dating, but also by histological dating and mid-luteal serum progesterone concentration at the time of sampling.

There are no data to suggest that a difference of 1 or 2 days is likely to have a major impact on the sex-steroid receptor expression. Indeed with the exception of one study looking at the PR and ER expression at LH+7 after treatment with ganirelix (Simon *et al.*, 2005), where no alteration in the steroid receptor expression was reported, data describing the steroid receptor expression in the mid-luteal phase after treatment with rFSH and a GnRH antagonist has not been previously reported. Luteal phase progesterone supplementation was administered to the oocyte donors (study group) to mimic an actual treatment cycle. Evidence exists to support the use of luteal phase progestogens after any form of down-regulation in an IVF/ICSI treatment cycle (Beckers *et al.*, 2003) and most centres now offer this routinely. Administering progesterone supplementation in one group (study) and not in another (control) may add an element of bias. However, the aim was to compare the differences in the mid-luteal phase endometrium from fertile untreated subjects, i.e. the physiological state with the mid-luteal endometrium of women routinely treated with a GnRH antagonist and rFSH.

This study has shown that under the effect of a GnRH antagonist and rFSH with luteal phase progesterone supplementation, there is a significant down-regulation of PR in the surface epithelium. The observation of PR down-regulation in the surface epithelium is in agreement with other studies evaluating PR content in the secretory phase of a normal menstrual cycle. It has been suggested that under the influence of progesterone, PR declines in the epithelium at the beginning of the

window of implantation (Lessey *et al.*, 1996). It therefore appears that any hormonal fluctuations that may result in the serum due to treatment with cetrorelix and Gonal-F® have little impact on intracellular PR in surface epithelium. In the secretory phase of a normal menstrual cycle, significant PR expression has been detected in the endometrial stroma (Lessey *et al.*, 1988). However, under the effect of a GnRH antagonist and rFSH and with progesterone supplementation, a significant down-regulation of PR in endometrial stroma was noted. Several genes have been localized to the endometrial stroma and significant proportions are expressed during the secretory phase (Yanaihara *et al.*, 2004). Studies have also shown an important role for progesterone in the endometrial stromal cell in induction of extracellular matrix in relation to implantation (Nakamoto *et al.*, 2005). A significant down-regulation of stromal PR could thus influence gene expression during the secretory phase and adversely impact endometrial receptivity.

PR protein has not been identified in the vascular endothelium (Krikun *et al.*, 2005), but PR are abundantly expressed in the perivascular cells throughout the menstrual cycle (Perrot-Applanat *et al.*, 1988; Critchley *et al.*, 2001). In this study, PR was significantly up-regulated in the perivascular cells of women treated with cetrorelix and Gonal-F®. Progesterone acting on the perivascular cells has been implicated in the modulation of endometrial blood flow. Furthermore, cytokine control in the perivascular cells is thought to be controlled by progesterone (Kelly *et al.*, 2002). These factors may well be important in the process of embryo implantation, and alterations of PR expression could influence these processes.

PR expression in the glands of untreated women was variable. In regular cycling women, in the secretory phase, significant PR content was maintained in the stroma, but diminished in the glandular epithelium (Lessey *et al.*, 1988). In this study, in some control biopsies, moderate glandular PR expression was maintained however, as evident in the box and whisker plot, there was marked variability in expression between biopsies. The number of women included in this study was small and this may have contributed to the apparent trend towards an increased mid-secretory PR expression in glands in untreated women. Furthermore, the women included in the control group were different from those who provided the

study biopsies in the treatment group. Whereas it would have been ideal to recruit the same oocyte donors as controls to further reduce the bias, practically it was only reasonable to seek an endometrial biopsy from an altruistic oocyte donor on a single occasion. In the control group, the mid-luteal phase was confirmed through consistency of reported LMP, circulating serum progesterone levels and histological dating. It is interesting to note that with rFSH and a GnRH antagonist treatment, glandular PR immunoexpression declined. This observation would be consistent with PR expression in the secretory phase of an untreated cycle.

PR mRNA levels are known to vary during the human menstrual cycle. In the late proliferative phase, glandular PR mRNA levels are significantly higher but it reduced in the secretory phase. In the stroma, PR mRNA remains unchanged (Lau *et al.*, 1996). Although there was some reduction in PR mRNA levels in the treatment group compared with the controls, the difference was not significant. However, PR changes in whole biopsies may not reflect the subtle changes in steroid receptor expression that exist between individual cell types.

In regular cycling women, ERα and ERβ have been identified in the endometrial epithelium, stroma, glands and perivascular cells (Saunders and Critchley, 2002). Only ERβ and not ERα is expressed in the vascular endothelium (Critchley *et al.*, 2001). No significant differences were observed in immunoexpression of either ERα or ERβ in treated women. Controlled ovarian stimulation is known to lead to supraphysiological levels of E2 and progesterone. These are thought to affect the endometrial receptivity through the predominantly progestational effects of endometrial phase advancement and premature luteinization (Kolb and Paulson, 1997). Hence, it appears that ER-mediated signalling appears to be of less importance in relation to implantation compared with PR mediated effects on the endometrium. It is interesting to note however that quantitative RT–PCR showed significantly reduced ERα mRNA levels in the treatment group.

AR expression is influenced by levels of circulating estrogens and androgens. Epithelial AR is up-regulated by estrogens and androgens and is inhibited by progestins (Slayden *et al.*, 2001b; Apparao *et al.*, 2002). AR is expressed in the endometrial stromal cells. The intensity of expression declines from proliferative phase to mid-secretory phase. In late secretory phase, AR expression is diminished

in all cell types (Mertens *et al.*, 2001). No significant difference in protein expression of AR was observed in women treated with rFSH and a GnRH antagonist, however, QRT–PCR showed significantly reduced AR mRNA levels in the group of women treated with a GnRH antagonist and rFSH. So far, there are very limited data on effects of AR and AR-induced gene expression in humans. Studies in pigs show AR in the pig endometrium during the window of implantation and demonstrate the functional, albeit complex, interactions of androgens and estrogens in the regulation of uterine endometrial gene expression and cell growth in vitro (Kowalski *et al.*, 2004). Further studies are needed to evaluate AR-induced gene expression in humans and the potential impact on embryo implantation.

The 3βHSD is weakly expressed in the glandular epithelium of the proliferative phase and moderately expressed in the glandular epithelium of secretory phase of the endometrium (Rhee *et al.*, 2003). In this study, irrespective of presence or absence of treatment, immunoexpression of 3βHSD in the glands, surface epithelium and endothelium was at a low level. No immunoexpression was seen in stroma and perivascular cells. No significant difference was observed in endometrial 3βHSD immunoexpression between the two groups of subjects. This suggests that the pre-ovulatory supraphysiological levels of estrogen and progestogen that result from COH and use of GnRH antagonists do not lead to any significant alteration in the levels of 3βHSD protein during the window of implantation. The currently available antibody against 3βHSD recognizes both forms of human 3βHSD enzymes, types 1 and 2. Hence it is not possible to comment on changes in the amounts of 3βHSD1 protein. However, RNA studies indicate that 3βHSD1 mRNA transcripts may be changing. A significant reduction in 3βHSD1 mRNA was observed in women treated with a GnRH antagonist and rFSH. The 3βHSD1 is responsible for the conversion of inactive pregnenolone to active progesterone and of dehydroepiandrosterone to androstenedione. A reduction in 3βHSD1 transcripts will ultimately lead to a reduction of intracellular progesterone. In the presence of altered PR expression, as observed with immunohistochemistry, it is likely that the reduced ligand availability for binding to PR leads to a disturbance in the dynamics of ligand-receptor interaction. This

may affect progesterone mediated signalling pathways including alterations in gene expression profiles thereby affecting the receptivity of the endometrium.

The 17βHSD5 transforms androstenedione to testosterone and also progesterone to the inactive 20-hydroxyprogesterone, and in the endometrium its immunoexpression has been localized to the surface epithelium and the vascular endothelium (Pelletier *et al.*, 1999). The findings in this study are in agreement with previous reports. There was a moderate to intense 17βHSD5 immunoexpression in endometrial surface epithelium and vascular endothelium. However, in this study, moderate 17βHSD5 immunoexpression was evident in the endometrial glands. No immunoexpression was observed in stroma and negligible immunoexpression was observed in the perivascular cells. No significant difference was observed in the 17βHSD5 immunoexpression between the two groups of subjects. A significant reduction of 17βHSD2 mRNA was demonstrated in women treated with rFSH and a GnRH antagonist. Since 17βHSD2 is involved in the inactivation of E2 to estrone and converting androgens to less potent forms, it is likely that higher levels of intracellular E2 and androgens persist thereby further disturbing the balance between estrogen, progesterone and androgens. This may further affect the endometrial development leading to suboptimal endometrial receptivity.

One previous report compared the effects of a GnRH antagonist treatment on mid-luteal phase endometrium to the state of the mid-luteal endometrium in a natural ovulatory cycle (Simon *et al.*, 2005). That study only examined ER and PR expression 7 days after an LH surge. The GnRH antagonist used in that study was ganirelix. In the current study, cetrorelix was used and to my knowledge this is the first report describing the ER, PR and AR expression as well as the steroid metabolizing enzymes expression in a GnRH antagonist treated mid-luteal phase endometrium.

The reason, why so few studies have addressed the mechanisms of endometrial receptivity in IVF/ICSI cycles during the window of implantation is due to the difficulty in obtaining, for detailed studies, endometrial biopsies at this phase of the treatment. Hence, most of the relevant studies have been performed on only small numbers of subjects.

Conclusions

In summary, these are novel data describing the effects of a GnRH antagonist and rFSH on sex-steroid receptor and steroid metabolizing enzymes expression in mid-secretory phase endometrium. This study has shown that under the effect of rFSH and a GnRH antagonist with progesterone supplementation, significant alterations occur in endometrial intracrinology at a time when the endometrium would be expected to be most receptive for implantation. The changes observed and the putative alterations that might occur as a result of these changes are summarised in Tables 4.9 (a), (b) & (c). The impact of these observations on embryo–endometrial interaction requires further evaluation.

Table 4.9 Summary of alterations in the endometrium after exposure to cetrorelix, Gonal-F® and progesterone.

(a) Changes in protein expression

Endometrial compartment	Changes observed
Surface Epithelium	Decrease in PR
Glandular Epithelium	Decrease in PR
Stroma	Decrease in PR
Perivascular Cell	Increase in PR

(b) Changes in mRNA expression

Endometrial compartment	Changes observed
Endometrial cell	Decrease in AR mRNA expression Decrease in ERα mRNA expression

(c) Putative alterations in intracellular hormone concentrations as a result of observed changes

Endometrial compartment	Putative changes
Endometrial cell	**Decrease** in progesterone concentration **Increase** in estradiol concentration **Increase** in androgen concentration

CHAPTER 5

Endometrial expression of steroid receptors in postmenopausal hormone therapy users: relationship to bleeding patterns.

5.1 Introduction

Hormone replacement therapy (HRT) is used by peri and postmenopausal women for the relief of menopausal symptoms and the prevention of osteoporotic fractures. The main therapeutic benefit is derived from estrogen but progestogen is necessary to prevent endometrial hyperplasia and adenocarcinoma. The majority of postmenopausal women take a continuous combined estrogen plus progestogen therapy that aims to avoid any vaginal bleeding. However, unpredictable and unscheduled vaginal bleeding or spotting is a common side effect described by up to 60% of HRT users (al-Azzawi and Habiba, 1994), leading to discontinuation of therapy in up to one in three users (Limouzin-Lamothe, 1996). Over 30% of cyclic HRT users and nearly half of all continuous combined HRT users make at least one visit to their gynaecologist with irregular bleeding (Ettinger *et al.*, 1998). In the majority of cases no pathology is found (Elliott *et al.*, 2003). In the Women's Health Initiative study, a prospective double-blinded trial to assess the major health benefits and risks of the most commonly used combined hormone preparations in the USA, irregular bleeding was responsible for un-blinding in almost 40% of women assigned to receive estrogen plus progestogen therapy (Rossouw *et al.*, 2002).

Irregular vaginal bleeding tends to settle with prolonged use, but since many national guidelines now advise restricting use of HRT to less than 5 years, unscheduled bleeding is likely to continue to be a clinical problem. There are no established ways of predicting, avoiding or treating unscheduled bleeding on HRT (Mossa *et al.*, 2003; North American Menopause Society, 2003). Few studies have addressed the potential mechanisms of HRT-induced bleeding and the underlying mechanisms are poorly understood. Changes have been reported in endometrial vascular density and perivascular support as well as increased numbers of stromal natural killer cells and an altered balance of matrix metalloproteinases to their tissue inhibitors, which may contribute to vascular breakdown and unscheduled bleeding in some HRT users (Hickey *et al.*, 2005; Hickey *et al.*, 2008; Hickey *et al.*, 2006). Human endometrium is a target for steroid hormones. Endometrial shedding and repair are considered inflammatory processes and sex steroids and glucocorticoids, acting via their receptors, are thought to play a vital role in the

processes involved (Jabbour *et al.*, 2006). Sex steroid receptor expression undergoes significant changes in regular cycling women, after treatment with exogenous estrogens and progestogens and in the presence or absence of breakthrough bleeding (Jabbour *et al.*, 2006; Milling-Smith and Critchley, 2005). Both endometrial estrogen and progesterone receptors (ER and PR) are up-regulated in stromal and glandular cells during the proliferative phase and subsequently downregulated in the glandular compartment during the secretory phase (Chauchereau *et al.*, 1992). PR expression persists in the stromal cells in the secretory phase. PR protein has not been identified in the endometrial vascular endothelium (Krikun *et al.*, 2005). Progesterone receptors are, however, abundantly expressed in the perivascular endometrial cells throughout the cycle (Perrot-Applanat *et al.*, 1988; Critchley *et al.*, 2001). Both forms of the ER are expressed in the perivascular cells, but only ERβ is present in endometrial endothelial cells (Critchley *et al.*, 2001). Endometrial stromal and endothelial cells express the GR (Bamberger *et al.*, 2001; Henderson *et al.*, 2003). During the menstrual cycle, the AR is expressed in endometrial stromal cells and intensity of AR immunostaining is greater during the proliferative as compared to the secretory phase (Mertens *et al.*, 2001; Burton *et al.*, 2003; Slayden *et al.*, 2001b).

The aim of this study was to investigate whether a relationship exists between endometrial steroid receptor expression and bleeding patterns in HRT users.

5.2 Methods

The women included in this study represent a subset from a larger study of bleeding mechanisms on HRT from the Menopause Clinic at King Edward Memorial Hospital (KEMH, Perth, Western Australia) between 2003 and 2005 (Hickey *et al.*, 2005; Hickey *et al.*, 2006). The study was approved by the institutional ethics committee of King Edward Memorial Hospital, WA, Australia and all subjects provided informed consent. The endometrial samples for this study were obtained in the Menopause clinic at King Edward Memorial Hospital, Perth, Western Australia. Endometrial biopsies obtained with the Pipelle® endometrial sampling device (Pipelle de Cornier, Laboratoire CCD, Paris, France) were used. Biopsies were fixed immediately in 10% formalin for 18 hours and tissue was then

embedded in paraffin. Sections were cut at 5 μm. The sections were dried at 37°C overnight. All biopsies had a standard haematoxylin and eosin section submitted to an experienced histopathologist for classification according to Noyes criteria (Noyes *et al.*, 1950) and for identification of any pathological features. The immunohistochemical analysis of these biopsies was carried out in the Centre for Reproductive Biology, University of Edinburgh, UK.

Thirty-eight endometrial biopsies were obtained from 21 postmenopausal women. Biopsies were obtained from seven postmenopausal women with at least 12 months of amenorrhoea who had not been exposed to any hormonal treatments in the previous 90 days. These latter postmenopausal subjects, not receiving HRT, were included as controls for comparative purposes of the effects of HRT exposure on the postmenopausal endometrium. A further 31 biopsies were obtained from 14 postmenopausal women using continuous combined HRT. Eleven women gave multiple biopsies (Table 5.1). Multiple biopsies were obtained in order to compare sex steroid receptor expression during and outside a bleeding episode in the same subject.

Table 5.1 Detailed breakdown of numbers of biopsies provided by all women taking part in this study

	Total number of women (*n*)	**Total number of biopsies (*n*)**
Women providing one biopsy	10	10
Women providing two biopsies	5	10
Women providing three biopsies	6	18
Total	21	38

Biopsies with adequate tissue for analysis were selected to represent the clinical categories of no HRT use (Group 1), HRT use with no bleeding (Group 2), HRT use with irregular bleeding (Group 3) and HRT use with irregular bleeding biopsied during a bleeding episode (Group 4) (Table 5.2). One endometrial biopsy was excluded as this was reported to be a benign polyp. A detailed medical history including bleeding patterns and current and previous HRT use was obtained from all the women. Bleeding diaries were prospectively completed over a 90-day reference period and bleeding patterns classified as either 'no bleeding' or 'irregular bleeding'. Irregular bleeding was defined as any vaginal bleeding occurring in postmenopausal continuous combined HRT users after at least 3 months of HRT use. The study was restricted to those women using oral or transdermal HRT and the range of products used are documented in Tables 5.3 and 5.4. Women were excluded if they were taking any herbal or other therapy that might alter endometrial histology.

Compliance with the HRT medication was monitored during this period and women were excluded if they did not take their HRT preparation strictly according to the manufacturer's instructions (daily for oral preparations or twice-weekly for transdermal preparations). Those with irregular bleeding were asked to contact the research nurse during bleeding episodes so that biopsies could be obtained both during and outside bleeding episodes. Irregular bleeding in HRT users was investigated according to standard hospital protocols. In brief, a Pap smear was obtained and transvaginal pelvic ultrasound arranged. If the endometrial thickness was ≤6 mm, an endometrial biopsy was obtained to assess histology. If the endometrium was >6 mm, hysteroscopy and endometrial biopsy were performed.

Table 5.2 Group numbers and definitions

Group	Women (*n*)	Group Definitions			Biopsies (*n*)
		HRT treatment> 3 months	**History of unscheduled bleeding**	**Bleeding at the time of endometrial biopsy**	
1	7	No	No	No	7
2	8	Yes	No	No	9
3	9	Yes	Yes	No	12
4	9	Yes	Yes	Yes	10
Total					38

Table 5.3 Estrogen and progestogen components of hormone replacement therapy (HRT) preparations used

Estrogen	Biopsies (*n*)	Progestogen	Biopsies (*n*)
Oral estradiol 1mg	11	Duphaston 10 mg	4
Oral estradiol 2mg	5	Norethisterone acetate 250 μg	14
Transdermal estradiol 50μg	10	Cyproterone acetate 5mg	1
Premarin 0.625 mg	3	Provera 5mg	11
Premarin 1.25 mg	2	Provera 10 mg	1
Total number of biopsies from HRT users	31		31

Table 5.4 Details of HRT preparations used

Patient codes in all study groups	**Mode of delivery of HRT**	**Estrogen preparation used**	**Progesterone preparation used**
Group 1	Women in Group 1 were not using HRT at the time of biopsy		
Group 2	Women using HRT with no history of bleeding		
LYCA	Transdermal	Estradiol 50µg	Norethisterone 250µg
MAJO	Transdermal	Estradiol 50µg	Norethisterone 250µg
HIYO	Oral	Estradiol 2mg	Cyproterone acetate 5mg
JABO	Oral	Estradiol 1mg	MPA 5mg
JUTO	Transdermal	Estradiol 50µg	Norethisterone 250µg
ANFA	Oral	Estradiol 1mg	MPA 5mg
MABY	Transdermal	Estradiol 50µg	Norethisterone 250µg
SHVA	Oral	Conjugated Estrogen 0.625mg	MPA 10mg
Group 3	Women using HRT with a history of bleeding, Endometrial biopsy conducted out with a bleeding episode		
LYCA	Transdermal	Estradiol 50µg	Norethisterone 250µg
SASI	Transdermal	Estradiol 50µg	Norethisterone 250µg
HETU	Oral	Conjugated Estrogen 0.625mg	MPA 5mg
MAAN	Transdermal	Estradiol 50µg	Norethisterone 250µg
OLNE	Oral	Estradiol 1mg	MPA 5mg
BRAU	Oral	Conjugated Estrogen 1.25mg	MPA 5mg
LAEL	Oral	Estradiol 1mg	Dydrogesterone10mg
MAPA	Oral	Estradiol 1mg	Dydrogesterone10mg
DOBA	Oral	Estradiol 2mg	MPA 5mg
Group 4	Women using HRT with a history of bleeding, Endometrial biopsy conducted during a bleeding episode		
SASI	Transdermal	Estradiol 50µg	Norethisterone 250µg
LYCA	Transdermal	Estradiol 50µg	Norethisterone 250µg
OLNE	Oral	Estradiol 1mg	MPA 5mg
BRAU	Oral	Conjugated Estrogen 1.25mg	MPA 5mg
MAAN	Transdermal	Estradiol 50µg	Norethisterone 250µg
LAEL	Oral	Estradiol 1mg	Dydrogesterone10mg
HETU	Oral	Conjugated Estrogen 0.625mg	MPA 5mg
DOBA	Oral	Estradiol 2mg	MPA 5mg
MAPA	Oral	Estradiol 1mg	Dydrogesterone10mg

MPA – Medroxyprogesterone acetate

5.2.1 Immunohistochemistry

Immunostaining for the tissue localisation of each steroid receptor was performed separately. Standard immunohistochemistry protocols were used as described in section 3. Paraffin-embedded tissue sections were dewaxed, rehydrated and an antigen retrieval step performed to expose the epitope (steroid receptor protein) under study. The sections were transferred into a Bond® automated immunohistochemistry staining system (Vision BioSystems, Newcastle upon Tyne, UK). The immunostaining procedures were performed as described previously (Henderson *et al.*, 2003; Burton *et al.*, 2003). The antigen-antibody complex was detected using a high-contrast polymer that included diaminobenzidine to identify positive immunoreactivity as indicated by presence of brown staining on tissue sections. Table 5.5 summarises the conditions for immunolocalisation of each of the epitopes studied.

Table 5.5 Immunohistochemistry protocols

Protein of interest	**Antigen Retrieval**	**Primary Antibody**	**Negative control**
Progesterone receptor (PR)	Pressure-cook Buffer – 0.01M Na Citrate pH 6.0	Monoclonal mouse anti PR antibody (Novocastra, Newcastle, UK) 1:400 in Bond Antibody diluent	Mouse Immuno-globulin IgG (Sigma,Dorset,UK) 1:8000 in Bond Antibody diluent
Glucocorticoid receptor (GR)	Pressure-cook Buffer – 0.01M Na Citrate pH 6.0	Monoclonal mouse anti GR antibody (Novocastra, Newcastle, UK) 1:40 in Bond Antibody diluent	Mouse Immuno-globulin IgG (Sigma,Dorset,UK) 1:320 in Bond Antibody diluent
Androgen receptor (AR)	Pressure-cook Buffer – 0.01M Na Citrate pH 6.0	Monoclonal mouse anti AR antibody (Biogenex, CA, USA) 1:360 in Bond Antibody diluent	Mouse Immuno-globulin IgG (Sigma,Dorset,UK) 1:450 in Bond Antibody diluent
Estrogen receptor alpha (ERα)	Pressure-cook Buffer – 0.01M Na Citrate pH 6.0	Monoclonal mouse anti ERα antibody (Dako,Cambridge, UK) 1:1200 in Bond Antibody diluent	Mouse Immuno-globulin IgG (Sigma,Dorset,UK) 1:7200 in Bond Antibody diluent
Estrogen receptor beta (ERβ)	Pressure-cook Buffer – 0.05M Glycine/0.01% EDTA	Monoclonal mouse anti ERβ antibody (Serotec, Oxford, UK) 1:40 in Bond Antibody diluent	Bond Antibody Diluent - Specificity of antibody as previously reported (Henderson *et al.*, 2003)

EDTA, ethylenediaminotetraacetic acid

Scoring and analysis of immunoreactivity

The immunostaining intensity of epitopes in all tissue sections was assessed in a standard semi-quantitative manner on a four-point scale: 0 = no immunostaining, 1 = mild immunostaining, 2 = moderate immunostaining and 3 = intense immunostaining. All tissue sections were scored blind by two observers. This scoring system has been previously validated in a subset of tissue sections in which immunoreactivity was measured with a computerized image analysis system and a strong correlation between quantitative data derived from image analysis and subjective scores by a trained observer was obtained (Wang *et al.*, 1998).

Statistics

Multiple measurements on the same subjects in the same group were averaged before analysis. Wilcoxon signed-ranks tests were used for comparisons between Groups 3 and 4 for which the measurements were made on biopsies from the same subjects, while the other five pair-wise comparisons of groups (i.e. Group 1 vs Group 2, Group 1 vs Group 3, etc.) were made using Mann-Whitney tests since all subjects in Groups 1 and 2 differed from all those in Groups 3 and 4. Two subjects contributed data to both Groups 1 and 2, and their measurements were excluded from the comparison of these two groups. Dunn's multiple comparison correction was then applied to the p values from each set of six tests.

5.3 Results

Endometrial biopsies were separated into four groups on the basis of bleeding patterns. Of the 38 biopsies analysed, seven were from postmenopausal women not using HRT (18%, Group 1), nine were from continuous combined HRT users with no bleeding in the previous 3 months (24%, Group 2), 12 were from continuous combined HRT users with a history of irregular bleeding in the previous 3 months (31%, Group 3) and 10 were from HRT users with irregular bleeding biopsied during a bleeding episode (26%, Group 4) (Table 5.2). The median age of women not using HRT was 52 years (interquartile range (IQR) 45–57, range 47–67 years). The median age of women on HRT was 54 years (IQR 53–57, range 42–62 years) and the median duration of HRT use was 34 months. There were no statistically

significant differences between the ages of HRT users and non-users (p = 0.445), time since menopause or body mass index of women between the groups. All HRT users were taking continuous combined HRT but as in normal clinical practice they were using a range of products (Table 5.4).

5.3.1 Endometrial histology

Of the 38 biopsies examined, 22/38 (58%) were classified as weakly proliferative, 14/38 (37%) were classified as atrophic, one appeared decidual and one appeared secretory. No relationship was observed between the histological appearance and bleeding patterns or to the type of estrogen or progestogen in the HRT used.

5.3.2 Immunohistochemistry

Immunoexpression of PR, GR, AR, ERα and ERβ at five cellular locations (i.e. in the endometrial glands, stroma, surface epithelium, vascular endothelium and in perivascular cells) was examined.

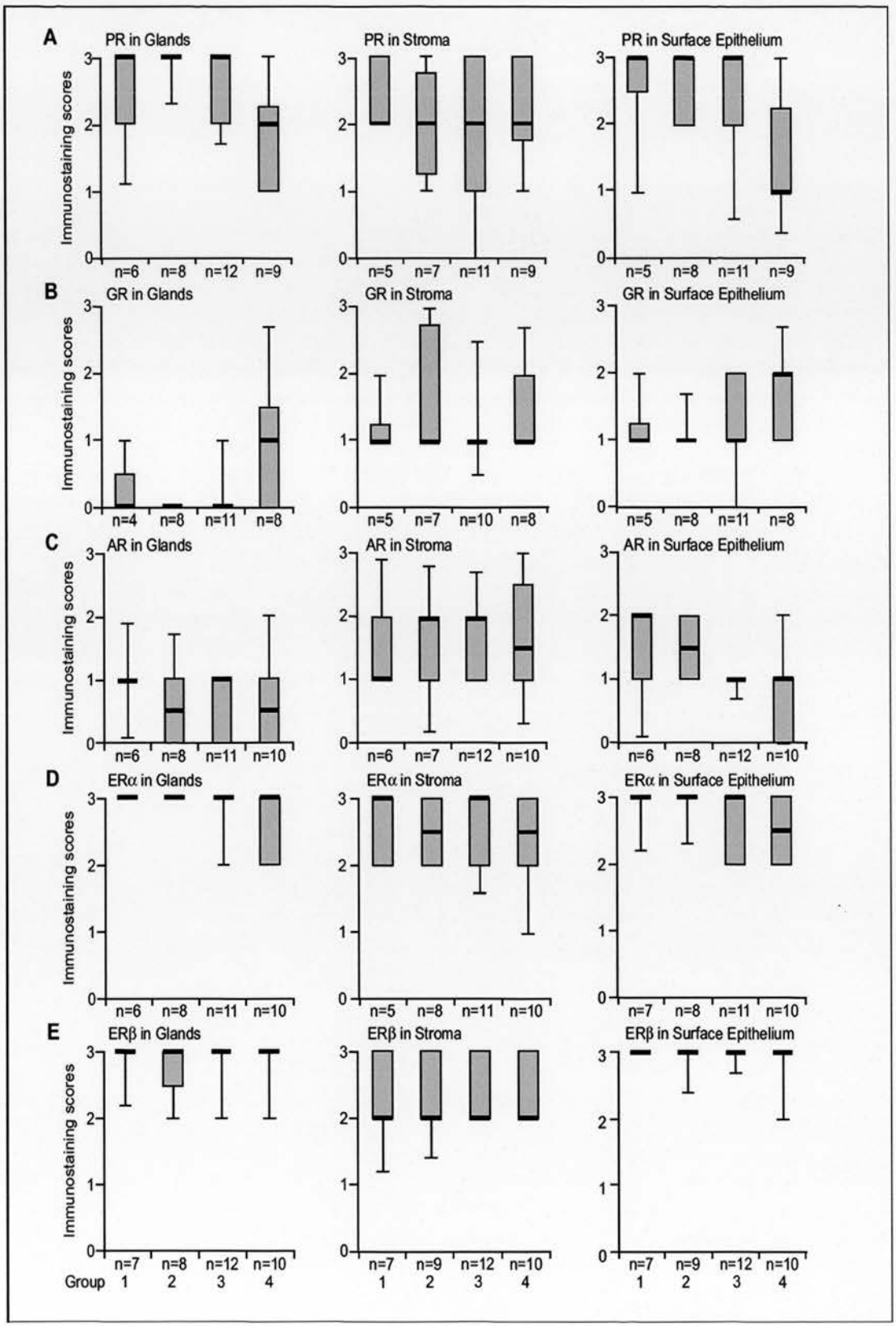
PR immunoexpression (Figures 5.1, 5.2(a), Table 5.6)

No PR expression was observed in the vascular endothelium and perivascular cells in any of the biopsies.

In the absence of HRT: moderate to intense PR expression was seen in the endometrial glands, stroma and surface epithelium.

In the HRT-treated endometrium: in all endometrial biopsies performed outside a bleeding episode, strong immunoexpression was observed in the endometrial glands, stroma and surface epithelium. The least amount of glandular PR expression was observed in women where the endometrial biopsy was taken during a bleeding episode. In comparison to biopsies from women with no bleeding, biopsies performed during a bleeding episode showed a strong trend (non-significant) towards down-regulation of endometrial glandular PR expression (p = 0.064). The surface and glandular epithelia showed identical trends, while the stroma showed no differences between groups.

Figure 5.1 Semi-quantitative immunoreactivity scores in endometrial cellular compartments in 4 groups of women. Box-and-whisker plots: box represents the 25th and 75th percentiles, whiskers represent the 10th and 90th percentiles and the heavy bar represents the median. NB: The *n* numbers represent the total number of biopsies studied in each group. The *n* numbers vary because in some biopsies that particular cell type was not reliably identified.


A
PR in Glands
PR in Stroma
PR in Surface Epithelium
B
GR in Glands
GR in Stroma
GR in Surface Epithelium
C
AR in Glands
AR in Stroma
AR in Surface Epithelium
D
ERα in Glands
ERα in Stroma
ERα in Surface Epithelium
E
ERβ in Glands
ERβ in Stroma
ERβ in Surface Epithelium
Immunostaining scores
0
1
2
3
n=6 n=8 n=12 n=9
n=5 n=7 n=11 n=9
n=5 n=8 n=11 n=9
n=4 n=8 n=11 n=8
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n=7 n=8 n=12 n=10
n=7 n=9 n=12 n=10
n=7 n=9 n=12 n=10
Group 1 2 3 4
1 2 3 4
1 2 3 4

Figure 5.2(a) Immunohistochemical localisation of steroid receptors in human menopausal endometrium. Positive immunoreactivity showing localisation of steroid receptor protein is indicated by brown staining. (A) PR immunostaining in endometrium from women on HRT with no bleeding (Group 2). Note high level of glandular immunoreactivity. (A) Inset: PR negative control - no immunoreactivity. (B) PR immunostaining in endometrium from women on HRT with bleeding, biopsy conducted during a bleeding episode (Group 4) demonstrating decreased glandular immunoreactivity. (C) GR immunostaining in endometrium from women on HRT with no bleeding (Group 2) illustrating down-regulation of glandular immunoreactivity. (C) Inset: GR negative control – no immunoreactivity. (D) GR immunostaining in endometrium from women on HRT with bleeding, biopsy conducted during a bleeding episode (Group 4) displaying increased glandular immunoreactivity. Note positive immunostaining in endothelial cells (arrow).
Scale Bar = 50 microns. G=Glands, S=Stroma, SE=Surface Epithelium

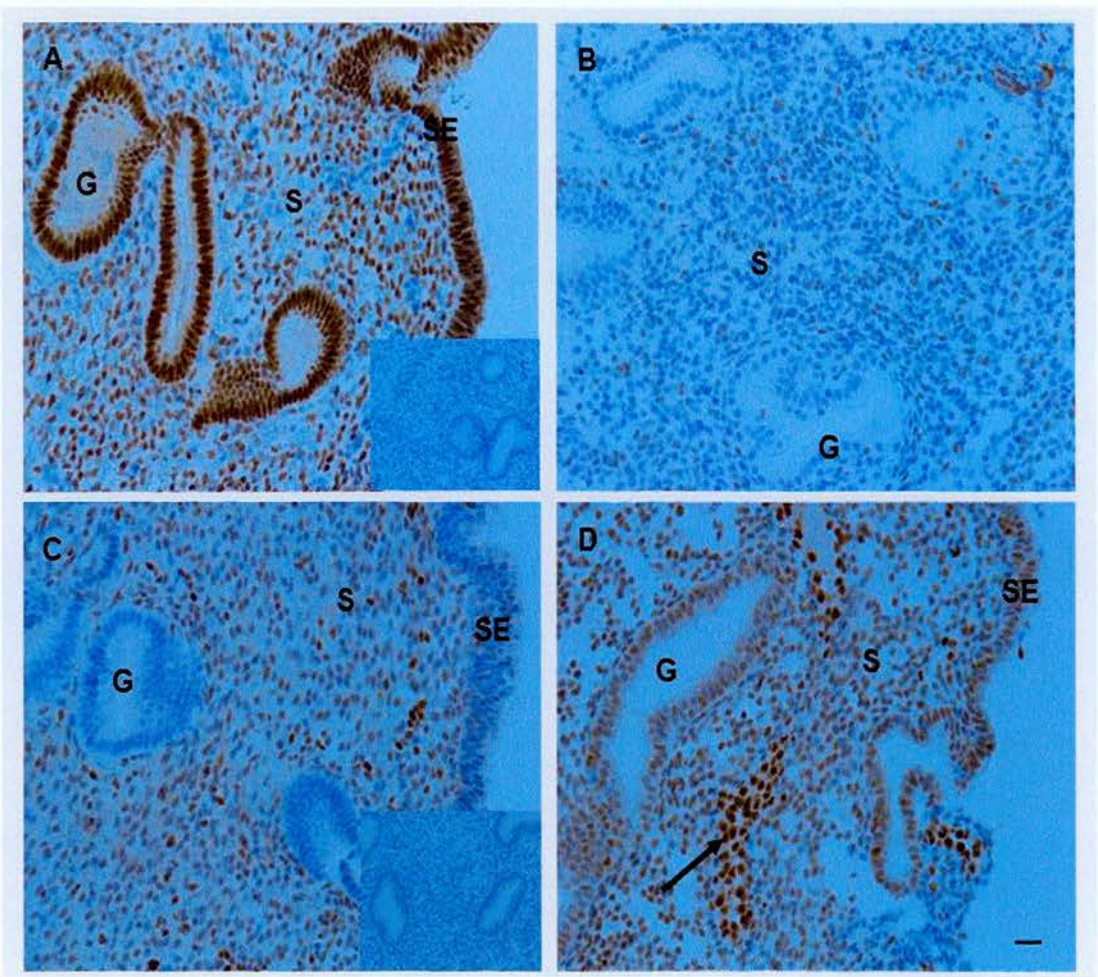


Figure 5.2(b) Immunohistochemical localisation of steroid receptors in human menopausal endometrium. Positive immunoreactivity showing localisation of steroid receptor protein is indicated by brown staining. (A) AR immunostaining in endometrium from women not on HRT with no bleeding (Group 1). Note low level of immunoreactivity in glands and stroma. (A) Inset: AR negative control - no immunoreactivity. (B) AR immunostaining in endometrium from women on HRT with bleeding, biopsy conducted during a bleeding episode (Group 4) illustrating low immunoreactivity in glands, stroma and surface epithelium.
Scale Bar = 50 microns. G=Glands, S=Stroma, SE=Surface Epithelium

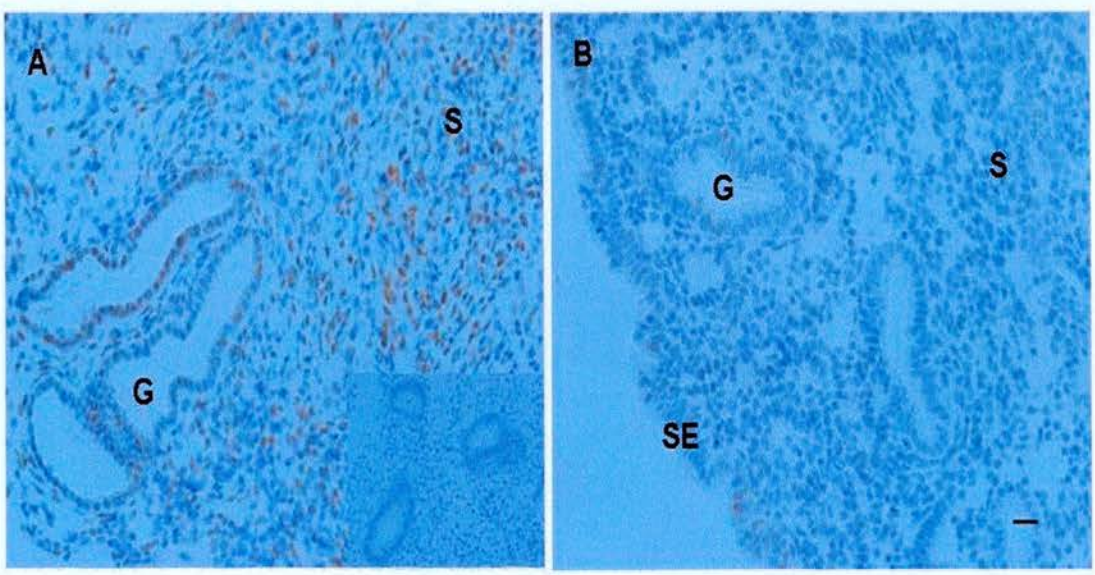


Table 5.6 Values of p from the 90 statistical tests performed[a]

Tissue	Comparison	PR	GR	AR	ERα	ERβ
Glandular	1 v 2	0.90	1.00	0.29	1.00	0.69
	1 v 3	0.86	0.94	0.39	0.53	0.76
	1 v 4	0.44	0.28	0.46	0.33	0.47
	2 v 3	0.54	0.47	0.68	0.47	0.96
	2 v 4	0.064*	0.087*	0.68	0.30	0.81
	3 v 4	0.256*	0.10	0.93	0.70	0.70
Stroma	1 v 2	0.34	0.20	0.89	0.73	0.66
	1 v 3	0.36	0.90	0.46	0.90	0.92
	1 v 4	0.61	0.62	0.95	0.61	0.76
	2 v 3	0.95	0.27	0.69	0.84	0.74
	2 v 4	0.69	0.57	0.95	0.76	0.81
	3 v 4	0.47	0.58	0.52	0.46	0.18
Surface Epithelium	1 v 2	0.41	0.73	0.90	1.00	1.00
	1 v 3	0.44	0.90	0.09	0.35	0.84
	1 v 4	0.11	0.22	0.18	0.21	0.84
	2 v 3	0.68	0.76	0.11	0.35	0.84
	2 v 4	0.06	0.12	0.14	0.21	0.76
	3 v 4	0.12	0.17	0.74	0.76	0.32

[a]Figures shown are uncorrected P-values from Mann-Whitney or Wilcoxon rank sum tests comparing each pair of study groups except for those asterisked (*) for which Dunn's correction was applied because the uncorrected p values were significant.

AR, androgen receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β; GR, glucocorticoid receptor; PR, progesterone receptor

GR immunoexpression (Figures 5.1, 5.2(a), Table 5.6)

In the absence of HRT: GR expression in the endometrial glands was negligible. Mild to moderate GR immunoreactivity was observed in the stroma, endothelium and surface epithelium.

In the HRT-treated endometrium: In biopsies obtained during a bleeding episode, a non-significant trend towards greater GR expression was observed in the endometrial glands compared to subjects with no bleeding ($p = 0.087$). The stromal expression of GR was variable. The immunoexpression in surface epithelium was mild to moderate in all groups of women, and more intense in the endothelium. Presence or absence of HRT, vaginal bleeding and the timing of the biopsy had no impact on the GR immunoexpression in the vascular endothelium and surface epithelium.

AR immunoexpression (Figures 5.1, 5.2(b), Table 5.6)

In all the biopsies, AR expression in the endometrial perivascular cells and vascular endothelium was negligible.

In the absence of HRT: mild AR expression was observed in the endometrial glands and stroma. AR expression in the surface epithelium was moderate.

In the HRT-treated endometrium: AR expression in the endometrial glandular epithelium and stroma in all groups of women was consistently at a low level.

Estrogen receptor α and β expression (Figures 5.1, Table 5.6)

In the absence of HRT: moderate to intense immunoreactivity for ERα and ERβ was observed in the endometrial glands, stroma, perivascular cells and surface epithelium. Only ERβ immunostaining was present in vascular endothelial cells.

In the HRT-treated endometrium: In all the groups of women, consistently strong ERα and ERβ expression was observed in the endometrial glandular epithelium, stroma and the surface epithelium.

5.4 Discussion

This study provides a detailed description of PR, GR, AR, ERα and ERβ receptor protein expression in endometrial cell components (glands, stroma, surface epithelium, perivascular and endothelial cells) of postmenopausal HRT users. Steroid receptor expression has been described in the context of bleeding patterns reported by subjects. No significant differences in steroid receptor expression in endometrium of women using HRT who report unscheduled bleeding episodes were demonstrated. Interesting trends (non-significant) in endometrial expression of glandular PR (decrease) and GR (increase) in postmenopausal HRT users who report unscheduled bleeding have however been observed. These patterns of steroid receptor expression in HRT users (with and without unscheduled bleeding) differ from those seen in premenopausal women using progestogen-only contraception, with bleeding from an apparently 'atrophic' endometrium. Different mechanisms are therefore likely to underlie abnormal bleeding in postmenopausal HRT users.

Perhaps because of technical difficulties in obtaining endometrial samples in the menopause, is there a paucity of data on steroid receptor expression in postmenopausal endometrium. Comparatively, pre-menopausal endometrium has been better characterized. Ideally it would not be appropriate to compare pre-menopausal endometrium with postmenopausal endometrium due to a fundamentally different hormonal milieu. However, in the absence of postmenopausal data, the only comparisons that can be made are in situations where the postmenopausal hormonal manipulation is likely to lead to an equivalent pre-menopausal state. There are data to suggest that continuous combined HRT use leads to a predominantly progestogenic effect on the endometrium (Wells *et al.*, 2002). The equivalent pre-menopausal endometrial phase would be the secretory phase.

All HRT users in this study were using continuous combined HRT. Therefore, it would be expected to find similar steroid receptor changes as those in the secretory phase of the normal menstrual cycle. In the latter, there is a down-regulation of ERα in the glands and stromal cells and of PR in glandular epithelium (Critchley *et al.*, 2001). The endogenous and exogenous hormonal milieu in postmenopausal HRT users is, however, quite different from that seen in the secretory phase in

premenopausal women where the specific sequential pattern of ovarian steroid production is tightly regulated. An interesting, albeit non-significant, observation was that with HRT, strong immunoexpression of PR in endometrial glands was maintained but in the presence of reported unscheduled bleeding glandular PR expression declined. Unlike the premenopausal state, in the postmenopausal untreated endometrium, strong glandular PR expression is maintained. In the absence of significant circulating levels of progestogens, the progestogenic stimulus to the endometrium is negligible. In regular cycling women, PR is down-regulated in the glandular compartment during the secretory phase by progesterone acting at both the transcriptional and post-transcriptional level (Jabbour *et al.*, 2006; Chauchereau *et al.*, 1992). Since these were observational studies, it is not possible to confirm whether the reduction in PR was a cause or a result of unscheduled bleeding, or if present whether the PR is functional. In premenopausal women using long-acting progestogen-only contraceptives (POC), a decrease in PR is observed even after short-term use of progestogen but this has not been shown to relate to bleeding patterns (Critchley *et al.*, 1998b; Hurskainen *et al.*, 2000). In depot medroxyprogesterone acetate users, stromal PR is reported to be reduced in those with irregular bleeding (Chotnopparatpattara *et al.*, 2003). Another interesting observation was the absence of PR expression in the perivascular cells in all studied biopsies. No perivascular PR expression was observed irrespective of the presence or absence of HRT and with or without bleeding. This observation appears to be a feature of postmenopausal endometrium in contrast to premenopausal endometrium since, in the premenopausal state, at least in women with regular cycles, progesterone receptors are abundantly expressed in the perivascular endometrial cells throughout the cycle (Perrot-Applanat *et al.*, 1988; Critchley *et al.*, 2001).

The mechanisms of bleeding with both POC and HRT are not fully understood but the endometrial changes associated with bleeding in HRT users appear to show some differences to those seen in younger women with progestogen-related irregular bleeding, suggesting that different mechanisms may be involved (Hickey *et al.*, 2006; Hickey *et al.*, 2003). Dahmoun and colleagues have reported that PR expression in the epithelium and stroma is unchanged before and during HRT

administration in subjects with no bleeding (Dahmoun *et al.*, 2004). The findings in the present study are in agreement with this report, with an added advantage of samples from subjects with irregular bleeding patterns and also biopsies specifically obtained during a bleeding episode. The results from the present study thus add to the body of knowledge that bleeding episodes in HRT users are associated with distinct endometrial changes. Changes have previously been reported in endometrial leukocyte populations during bleeding in HRT users with an increase in uterine natural killer cells (Hickey *et al.*, 2005) and changes in stromal expression of matrix metalloproteinases and their tissue inhibitors (Hickey *et al.*, 2006; Hickey *et al.*, 2001).

GR expression in endometrium is normally localised to stromal and endothelial cells (Bamberger *et al.*, 2001; Henderson *et al.*, 2003). The role for GR and glucocorticoids in human endometrium has not been defined. Increased GR expression may potentiate effects of endogenous/locally derived cortisol thus suppressing angiogenesis (Narvekar *et al.*, 2006; Small *et al.*, 2005) and aggravating potential for unscheduled bleeding. Augmented local cortisol action may also disturb local endometrial prostaglandin production. Prostaglandins play an important role in endometrial bleeding (Baird *et al.*, 1996). Disturbance in the balance of local factors maintaining endometrial blood vessel integrity and stability could be another possible mechanism responsible for unscheduled bleeding.

In postmenopausal endometrium studied here there were no differences in AR or ER (α and β) expression, irrespective of presence or absence of HRT and bleeding pattern. In postmenopausal untreated endometrium, Dahmoun and colleagues observed high levels of ERα expression in glandular epithelium but with HRT treatment a decrease in expression was seen (Dahmoun *et al.*, 2004). In the present study, no down-regulation of expression of ERα or ERβ in either glandular or stromal cells was observed after HRT exposure. Presence or absence of bleeding also did not make any significant difference to endometrial ER (ERα or ERβ) expression. Dahmoun and colleagues used only two preparations of HRT in their study (Dahmoun *et al.*, 2004). Five different continuous combined HRT preparations were used as a part of this study. This may have contributed to the differences in these findings. Alternatively it may suggest variable functional

alterations of ER and PR in the postmenopausal endometrium during exposure to differing hormonal preparations.

It is acknowledged that the sample size in this present study is small. A reason why so few studies have addressed the mechanisms of irregular bleeding in HRT users is that adequate endometrial samples from postmenopausal women with no endometrial pathology and a thin endometrium can be extremely difficult to obtain. Furthermore, obtaining samples during a bleeding episode requires particular vigilance and a high level of compliance amongst postmenopausal women.

A variety of HRT preparations were used by the women taking part in this study. This may have influenced the endometrial cellular response. Nevertheless, irregular bleeding is common to all HRT preparations and hence it is reasonable to work on the assumption that common mechanisms may underlie these phenomena.

Conclusions

The present study of endometrial steroid receptor expression and bleeding patterns in HRT users has failed to demonstrate significant differences in steroid receptor protein expression in those women who report unscheduled bleeding episodes. These observations differ from the endometrial steroid receptor expression observed with normal menstruation and long-term progestogen-only administration, suggesting that different local mechanisms are involved in HRT-related bleedings. Interesting trends (non-significant) however have been observed in endometrial expression of glandular PR (decrease) and GR (increase) in postmenopausal HRT users who report unscheduled bleeding. These observations warrant further investigation among a larger group of HRT users with an endometrial biopsy during a bleeding episode to explore the mechanisms underlying problematic unscheduled bleeding. It is unlikely that effective strategies for the treatment or prevention of abnormal bleeding on HRT will be developed unless the underlying local mechanisms are understood.

CHAPTER 6

Intrauterine release of progesterone antagonist ZK230211 is feasible and results in novel endometrial effects: a pilot study

6.1 Introduction

Unscheduled breakthrough bleeding (BTB) associated with the use of progestin-only contraception remains a major problem, often resulting in poor compliance and discontinuation of progestin-only contraceptive methods (Dugoff *et al.*, 1995; Kovacs, 1996). Intrauterine release of levonorgestrel (LNG) by means of the LNG-releasing -intrauterine system (LNG-IUS) is highly effective for contraception as well as for the treatment of heavy menstrual bleeding (menorrhagia) (Andersson *et al.*, 1994; Luukkainen and Toivonen, 1995; Hurskainen *et al.*, 2004). However, initiation of LNG-IUS treatment is associated with a high incidence of BTB, which typically resolves following the first few months of LNG-IUS use (Andersson *et al.*, 1994; Hurskainen *et al.*, 2004).

The effects on the endometrium of continuous administration of progesterone antagonists (PAs) such as mifepristone and ZK137316 have been evaluated both in women (Baird *et al.*, 2003a; Brown *et al.*, 2002; Narvekar *et al.*, 2004) and in non-human primates (Wolf *et al.*, 1989; Slayden and Brenner, 1994; Slayden *et al.*, 1998; Slayden *et al.*, 2001; Chwalisz *et al.*, 2000). In humans, daily administration of mifepristone in the doses of 2mg and 5mg was shown to induce amenorrhoea in majority of women taking part in the study over a period of 120 days. Ovulation was also suppressed in the majority of women however follicular activity was maintained (Brown *et al.*, 2002). Endometrial histology in the women taking part in this study showed inactive proliferative or cystic changes with dense stroma. There was a significant decrease in markers of proliferation, i.e. mitotic index and Ki67 staining (Baird *et al.*, 2003a). In a related study there was a significant decrease in the expression of H3 mitosis marker and progesterone receptor in endometrial glands and stroma by day 60 of treatment with 2mg and 5mg doses of mifepristone. In contrast, the expression of androgen receptor increased in glands, surface epithelium, and stroma compared with the pre-treatment endometrium. These changes were maintained at 120 days of treatment. The expression of estrogen receptor was unchanged in stroma and surface epithelium; however, there was a significant decrease in expression after 120 days of treatment. As androgens can antagonize estrogen action, enhanced glandular androgen receptor expression induced by mifepristone could play a role in its anti-proliferative effects (Narvekar

et al., 2004). Treatment with ZK137316 upregulated the PR expression. Furthermore, ZK137316, in the presence or absence of E_2, reduces VEGF release in fibroblast cell culture but it showed no significant effects in epithelial cell culture. The different results for the epithelial cells and fibroblasts indicate that the pharmacological effects of progesterone antagonists may be cell specific and depend on the presence or absence of partial progestogenic agonistic activities (Classen-Linke *et al.*, 2000).

In non-human primates, PAs inhibit endometrial proliferation and induce amenorrhoea (Wolf *et al.*, 1989; Slayden *et al.*, 1998; Slayden *et al.*, 2001). When administered chronically at relatively low doses, PAs block the mitotic activity of endometrial epithelium and induce stromal compaction in a dose-dependent manner in both spayed and intact monkeys (Wolf *et al.*, 1989; Slayden and Brenner, 1994; Slayden *et al.*, 1998; Slayden *et al.*, 2001; Heikinheimo *et al.*, 1996). As follicular development is not suppressed during PA administration (Heikinheimo *et al.*, 1995; Slayden *et al.*, 2001), endometrial suppression is not associated with a decrease in circulating levels of estradiol. Thus the mechanism of action of endometrial suppression remains somewhat enigmatic; atrophy of the uterine spiral arteries being a likely explanation (Chwalisz *et al.*, 2000).

The PA ZK230211 is a highly potent PA with no progestogenic effects (Fuhrmann *et al.*, 2000; Slayden *et al.*, 2001). Systemic daily administration of ZK230211 at doses of ≥16 μg/kg to cynomolgus monkeys suppresses ovulation and menstruation (Slayden *et al.*, 2001). Similarly, intrauterine release of 3–4 μg and 26–30 μg of ZK230211/24 h had an antiproliferative effect on primate endometrium (Nayak *et al.*, 2000). Thus intrauterine release of PAs, especially ZK230211, may make them useful in both contraception and hormone therapy for various gynaecological indications. Specifically, endometrial suppression by means of PA-releasing IUS might be effective in the treatment of heavy or prolonged uterine bleeding. Moreover, a PA-IUS is likely to convert the endometrium into a non-receptive state, which may be utilized in the development of novel contraceptive strategies.

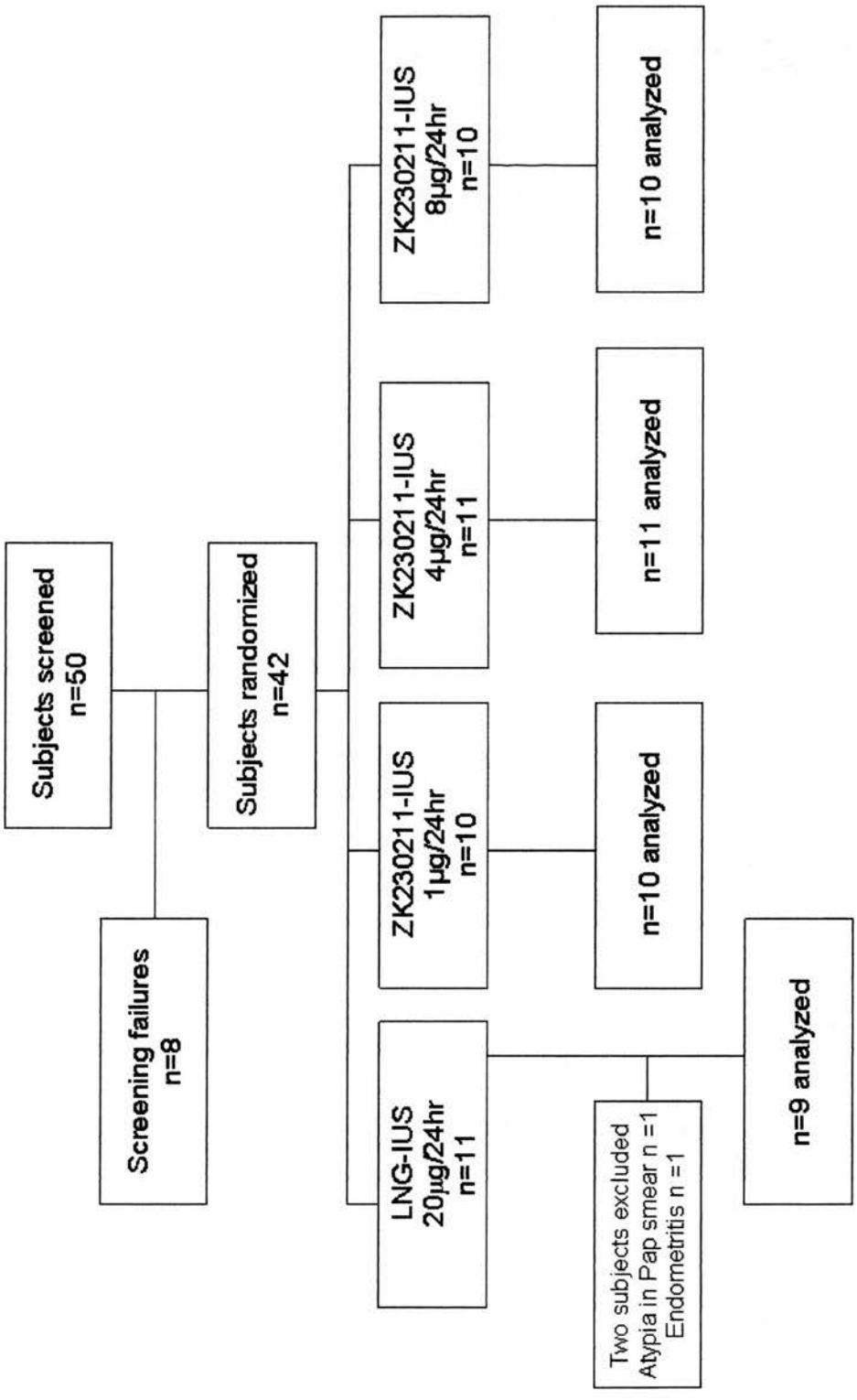
In the present randomized, single-blinded, prospective proof-of-concept trial, the bleeding patterns and endometrial effects of intrauterine release of the PA

ZK230211 versus progestin LNG were evaluated in women scheduled for hysterectomy owing to heavy or painful menstruation.

6.2 Methods

The study subjects were identified among women on waiting list for hysterectomy at the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital. The endometrial tissue samples were obtained from women at the time of a hysterectomy. Women scheduled for hysterectomy owing to idiopathic heavy menstrual bleeding (menorrhagia) or painful menstruation (dysmenorrhoea) participated in the study. The numbers of days of bleeding (requiring protection) and spotting (no protection required) during 30-day periods preceding insertion of the IUS, and preceding hysterectomy, were assessed from bleeding diaries. Prior to participation, each woman signed an informed consent document. The study protocol was approved by the Institutional Review Board of Helsinki University Central Hospital and the Finnish National Agency for Medicines. Figure 6.1 shows the flow of the subjects through the study.

Figure 6.1 Flow of the subjects through the study



The criteria for inclusion in the study were written informed consent, age 30–48 years, regular menstrual periods with cycle length between 21–35 days, length of the uterine cavity between 6 and 10 cm and good general health. The exclusion criteria were: endometrial polyps or hyperplasia, submucosal myoma or intramural myoma exceeding 4 cm in greatest diameter or myoma distorting the uterine cavity, an ovarian cyst exceeding 4 cm, epithelial cell atypia in the Pap smear, concomitant use of an intrauterine device, history of climacteric symptoms and systemic use of sex steroids within the last 3 months.

The experimental IUS's releasing ZK230211 (ZK-IUS) at rates of 1, 4 and 8 μg/24 h were provided by Schering Ag (Berlin, Germany). The LNG-releasing IUS (20 μg/24 h; MIRENA, Schering Oy, Turku, Finland) was used as a comparator. Similarly as for

LNG-IUS, the ZK-IUS consisted of a polyethylene body in the shape of T with a mixture of ZK230211 and polydimethyl siloxane mounted around the vertical arm. The dimensions of the ZK-IUS and LNG-IUS were similar (32 x 32 mm).

The women were randomized using SAS/PLAN by Schering Oy to any of the three experimental ZK-IUS's or LNG-IUS. Investigators had a separate stock of identically packaged IUS's and randomization numbers. However, the nature of the IUS became apparent to the study personnel following opening of the packaging. The IUS's were inserted between 4 and 8 weeks prior to scheduled hysterectomy. The duration of the trial was based on previous toxicological data obtained from non-human primates, with the drug regulatory agency permitting the study to last up to 8 weeks. Insertion was performed between days 1 and 7 of the menstrual cycle. Following insertion, fundal location of the IUS was verified by means of pelvic ultrasonography.

Of the 42 women randomized, 11 women (acting as controls) were randomized to receive LNG-IUS. Of these 2 women were excluded and hence 9 women provided endometrial tissue for the study. Of the women randomized to receive ZK230211-IUS, women using ZK230211-IUS 1μg (n=10), ZK230211-IUS 4μg (n=11) and ZK230211-IUS 8μg (n=10) provided samples for the study. On the morning of hysterectomy, the location of the IUS was again verified by means of pelvic ultrasonography.

The following specimens were collected just before and immediately after removal of the uterus at hysterectomy.

1. Just prior to the surgery, a venous blood sample was collected for determination of serum ZK230211 concentration.
2. Following surgery and collection of specimens for routine pathological examination, separate samples of endometrium were collected from the fundal, mid-corpus and isthmic areas of the uterus for haematoxylin and eosin staining and immunohistochemistry (IHC).
3. Thereafter, separate samples of endometrium and myometrium were taken for determination of ZK230211 levels. All the remaining endometrial tissue (after the histological samples were taken) was separated and collected for these measurements.

The primary outcome measure was assessment of endometrial morphology during the use of ZK-IUS in comparison to that of LNG-IUS. The secondary objectives included assessment of bleeding patterns, determination of uterine and serum concentration of ZK230211 and evaluation of the effect of ZK-IUS versus LNG-IUS on selected endometrial markers.

Serum and uterine tissue measurements of ZK230211

The samples were pre-prepared and sent for analysis to the analytical laboratory of Schering AG, Berlin, Germany. A liquid chromatography mass spectrometer/mass spectrometer method was used for the measurement of ZK230211 concentrations. A peripheral venous blood sample was obtained prior to surgery. The lowest limit of quantification in human serum samples was 50 pg/ml, and in the uterine tissue samples (endometrial and myometrial), it was 10–25 ng/g.

Endometrial morphology

Endometrial morphology was assessed from haematoxylin and eosin stained tissues taken from the fundus, corpus or isthmus. In addition to routine diagnostic analysis, estrogenic (E) and progestogenic (P) activities were evaluated by means of the following criteria: E0, atrophic glands; E1, small single-layered glands with rare mitosis; E2, moderately sized glands with two or three cell layers and occasional

mitoses; E3, tortuous glands, several cell layers, easily detectable mitotic activity; P0, no signs of a progestogenic effect; P1, basal vacuolization in most cells; P2, dilated glands with secretory activity; P3, stromal decidualization. The combined E and P score was assigned to one of the three categories: fully active = E2-3P0; partially suppressed = E2-3P1-3 or E1P0-3; or fully suppressed = E0. The samples were assessed independently by two pathologists. In cases of disagreement, a third pathologist was included. If the hormonal effect varied between anatomical locations, the overall result from two of the three locations (fundus, corpus or isthmus) was taken as the consensus score.

Immunohistochemistry

Standard immunohistochemistry protocols, as described in section 3.2.1, were followed. Tissue expression of androgen receptor (AR), progesterone receptor (PR) and estrogen receptors alpha and beta (ERα and ERβ) was assessed as described previously (Critchley *et al.*, 2001; Slayden *et al.*, 2001; Henderson *et al.*, 2003). The proliferation markers Ki-67 and phosphorylated histone H3 (PH3) were evaluated as described by Brenner et al. (Brenner *et al.*, 2003) and Narvekar et al. (Narvekar *et al.*, 2004). Finally, insulin-like growth factor-binding protein-1 (IGFBP-1) was evaluated according to Pekonen et al. (Pekonen *et al.*, 1992). The antigen retrieval methods, primary antibodies and negative controls used in the immunohistochemical analyses are summarized in Table 6.1. The amounts of the above-mentioned epitopes were assessed by two blinded observers in a semi quantitative manner on a 4-point scale: 0, no staining; 1, mild/minimal staining; 2, moderate immunostaining and 3, intense immunostaining.

Statistical analysis

Statistical analyses were performed using the chi-square test, the Fisher's exact test, the Mann–Whitney U-test or the Kruskal– Wallis test, as appropriate. A two-tailed P-value lower than 0.05 was considered statistically significant. The calculations were performed with StatView statistical software (SAS Institute Inc., Cary, NC, USA).

Table 6.1 Summary of the antigen retrieval methods, primary antibodies and negative controls used in various immunohistochemical analyses

Protein of interest	**Antigen retrieval**	**Primary Antibody(AB)**	**Negative control**
PR	Microwave Buffer – 0.01M Na Citrate	Monoclonal mouse anti-PR AB (Novocastra, Newcastle, UK) (1:40)	Mouse immunoglobulin (Ig) G (Sigma, Dorset, UK) (1:800)
ERα	Microwave Buffer – 0.01M Na Citrate	Monoclonal mouse anti-ERα AB (Dako, Cambridge, UK) (1:400)	Mouse immunoglobulin IgG (Sigma, Dorset, UK) (1:2400)
ERβ	Pressurecook Buffer – 0.05M glycine/0.01% EDTA	Monoclonal mouse anti-ERβ AB (Serotec, Oxford, UK) (1:40)	Bovine Serum Albumin
AR	Pressurecook Buffer – 0.01M Na Citrate	Monoclonal mouse anti-AR AB (Biogenex, CA, USA) (1:240)	Mouse immunoglobulin IgG (Sigma, Dorset, UK) (1:300)
Ki67	Microwave Buffer – 0.01M Na Citrate	Monoclonal mouse anti-Ki67 AB (Novocastra, Newcastle, UK) (1:50)	Mouse immunoglobulin IgG (Sigma, Dorset, UK) (1:500)
PH3	Pressurecook Buffer – 0.01M Na Citrate	Rabbit anti-phospho-histone AB (Upstate Biotech., Buckingham, UK) (1:1000)	Rabbit immunoglobulin IgG (Vector Lab., UK) (1:1000)
IGFBP-1	Vectastatin ABC kit (Vector Lab, CA, USA)	Monoclonal anti-IGFBP-1 AB (Mab 6303) (Medix Biochemical, Kauniainen, Finland) (1:1000)	Mouse immunoglobulin IgG (Vector Lab., CA, USA) (1:1000)

6.3 Results

Two women dropped out of the study, one because of endometritis following insertion of an LNG-IUS and another one because of epithelial cell atypia in the Pap smear collected prior to insertion of an IUS. Thus 40 subjects completed the study; their demographic details are shown in Table 6.2. The four groups did not differ from each other as regards age, body mass index or uterine weight. Most (93%) of the subjects were parous. Hysterectomy was performed abdominally in 5%, laparoscopically in 45% and vaginally in 50% of the cases.

Table 6.2 Characteristics of the study subjects
The data are presented as mean ± SD; BMI = Body Mass Index

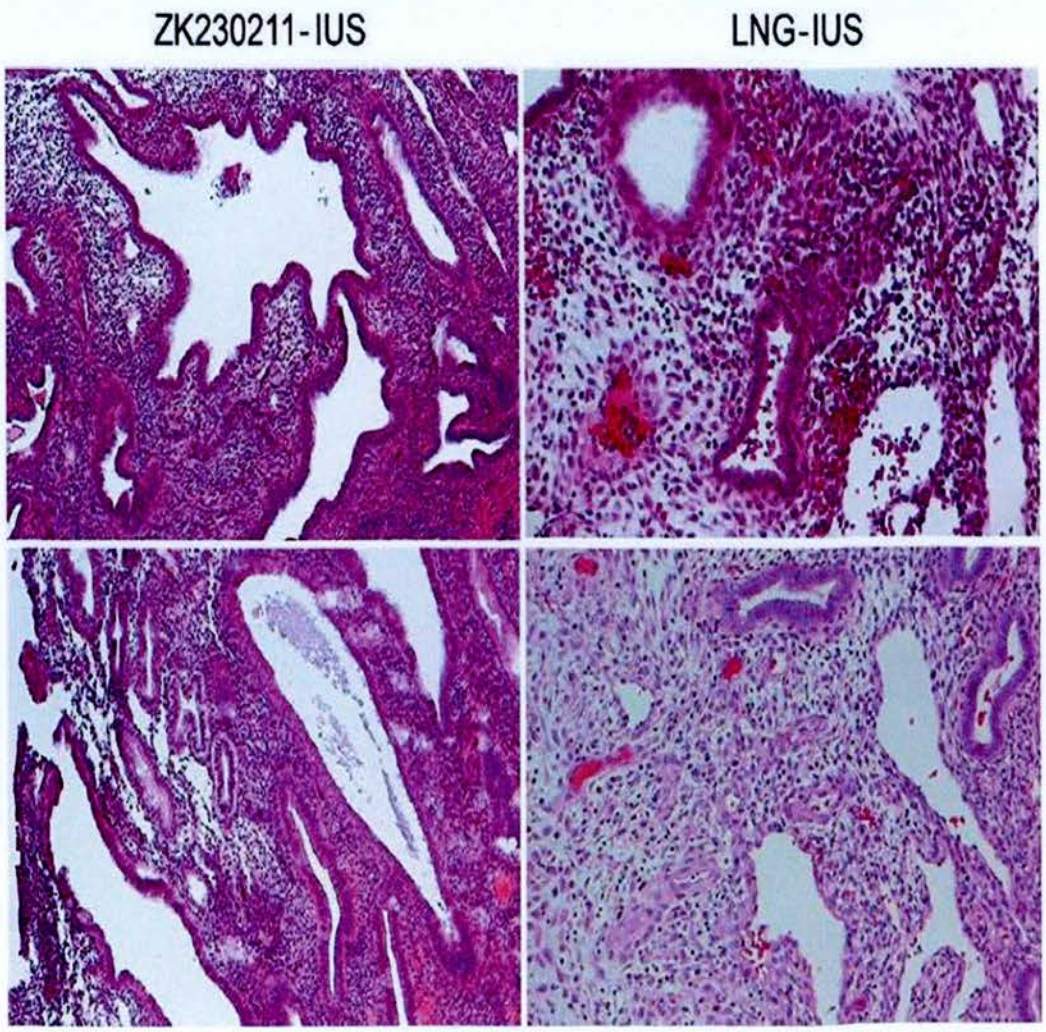
	ZK230211-releasing IUS			LNG-IUS
	1µg/24h	4µg/24h	8µg/24h	
n	10	11	10	9
Age (years)	43.4 ± 3.0	42.0 ± 5.3	43.7 ± 2.6	43.1 ± 2.3
BMI (kg/m^2)	25.8 ± 3.8	25.6 ± 4.9	25.0 ± 5.2	26.7 ± 4.2
Exposure time (days)	41.6 ± 6.9	41.2 ± 6.3	45.1 ± 7.7	44.3 ± 6.8
Uterine weight (g)	161 ± 31	147 ± 38	140 ± 60	145 ± 42

6.3.1 Endometrial morphology

The primary outcome measure was comparison of endometrial morphology. Figure 6.2 illustrates endometrial morphology after treatment with an IUS releasing 8 μg of ZK230211/24 h, or LNG (20 μg/24 h). Endometrial morphology differed in relation to ZK230211 and LNG treatment. In LNG-treated endometrium, marked stromal decidualization was seen and the morphology of the glands varied from inactive to secretory. On the other hand, stromal decidualization was not observed after ZK230211 treatment. The glands, in contrast, were often dilated and the epithelial cells showed little proliferative activity and a secretory morphology.

Endometrial morphology was judged to reflect partial suppression in ≥2 of the 3 locations (fundus, corpus and isthmus) in 30%, 9% and 10% of women with ZK-IUS's releasing 1, 4 and 8 μg/24 h, respectively, and in 67% of women with an LNG-IUS. The difference in endometrial morphology was statistically significant between the groups of women using the LNG-IUS versus ZK-IUS's releasing 4 and 8 μg/24 h ($P<0.01$ and $P<0.02$, respectively). The difference between women with an LNG-IUS versus a ZK-IUS releasing 1μg/ 24 h approached significance ($P = 0.11$). A high degree of morphologically estrogenic effects (grade 3 in 60–82%) were seen following the use of ZK-IUS's, whereas only 11% of the endometrial specimens collected following use of an LNG-IUS displayed grade 3 estrogenic effects. Moreover, the distribution of morphologically estrogenic effects (grade 1–2 versus 3) differed significantly between subjects using an LNG-IUS versus IUS's releasing 1, 4 and 8 μg of ZK230211/24 h ($P<0.05$, $P<0.002$ and $P<0.005$, respectively). However, morphologically progestogenic effects (grade 0–2 versus 3, or grade 0–1 versus 2–3) were not significantly different between the endometrial specimens exposed to an LNG-IUS or any of the ZK-IUS's.

Figure 6.2 Haematoxylin and eosin-stained human endometrium following treatment with an IUS releasing 8 μg of ZK230211/24 h (panels on the left), and treatment with an LNG-IUS (panels on the right). Note the stromal compaction and non-functional secretory morphology in the glands (Non-functional secretory morphology refers to the situation where the glands have a tortuous appearance similar to that seen in the secretory stage but lack the functional ability of secreting glandular secretions, as is evident particularly in the Top left figure) following ZK230211 treatment. In LNG-treated endometrium, marked stromal decidualization was seen and the morphology of the glands varied from inactive to secretory.



6.3.2 Bleeding patterns

The secondary objectives included assessment of bleeding patterns, determination of uterine and serum concentrations of ZK230211 and evaluation of the effect of ZK-IUS versus LNG-IUS on selected endometrial markers. Figure 6.3 shows the numbers of days of bleeding and spotting (mean + SD) during the 30-day periods immediately preceding insertion of the IUS's, and hysterectomy. The number of days with bleeding and spotting increased significantly in the group randomized to the LNG-IUS ($P<0.01$), whereas non-significant changes were observed in the three groups randomized to ZK-IUS's.

6.3.3 Uterine tissue and serum concentrations of ZK230211

ZK230211 was measurable in all endometrial specimens. The mean (+SD) endometrial concentrations of ZK230211 (per gram of tissue wet weight) were 83.7+40.6, 83.8+39.5 and 166.9+134.6 ng/g in the groups using ZK-IUS's releasing 1, 4 and 8 μg of ZK230211/24 h, respectively. Only four subjects (one in each group using a ZK-IUS releasing 1 and 4 mg of ZK230211, and two in the group using an IUS releasing 8 mg of ZK230211) had measurable levels of ZK230211 in the myometrium. The individual concentrations varied from 8.4 to 47.7 ng/g of myometrial-wet weight. However, serum concentrations of ZK230211 were below the quantification limit of the assay in all samples analysed (n = 31) (Table 6.3).

Figure 6.3 Days of bleeding and spotting (mean + SD) during a 30-day period preceding (B) insertion of a ZK230211-IUS or an LNG-IUS, and preceding hysterectomy (D) in the four experimental groups. The number of days of spotting and bleeding increased significantly in the LNG-IUS group ($P < 0.01$)

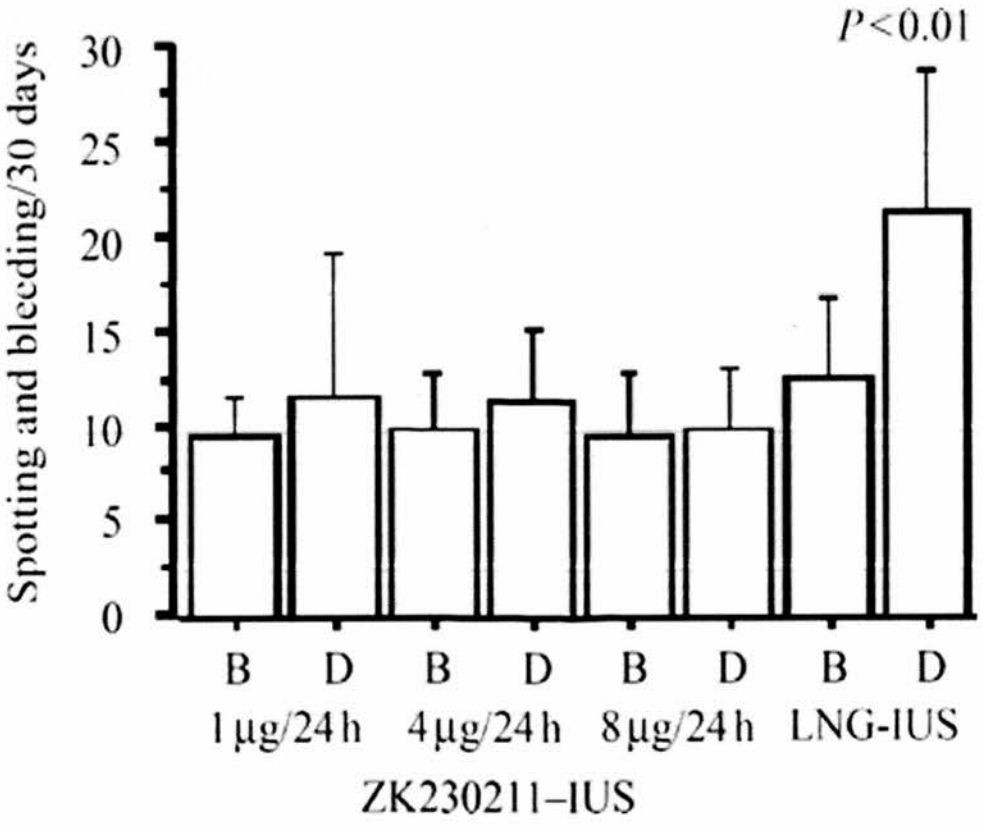


Table 6.3 Endometrial content of ZK230211 (per g of tissue wet weight) (mean ±SD)

Endometrial content of ZK230211 (per g of tissue wet weight) (mean ±SD)		
	Release rate of ZK230211	Concentration of ZK230211
	1μg/24 h	83.7 ± 40.6 ng/g
	4μg/24 h	83.8 ± 39.5 ng/g
	8μg/24 h	166.9 ± 134.6 ng/g
	ZK230211 was not measurable in any of the serum samples	

6.3.4 Immunohistochemistry

Figures 6.4 and 6.5 show a summary of the various immunohistochemical analyses performed. Staining for sex steroid receptors PR, ERα, ERβ and AR was analysed in endometrial glands, stroma and surface epithelium. In addition, staining for proliferation markers phosphorylated Histone 3 and Ki-67 and decidualisation marker IGFBP-1 was analysed separately.

Sex steroid receptors

Immunostaining for PR in endometria exposed to intrauterine ZK230211 and LNG is illustrated in Fig. 6.4A and 6.4B, respectively. PR was not detectable in the surface epithelium following exposure to intrauterine LNG. It was, however, detectable in the surface epithelium following exposure to ZK230211; the difference was significant between the groups exposed to LNG and all three doses of ZK230211 ($P<0.01$ – <0.05). In endometrial glands and stroma, intense immunoreactivity for PR was observed after intrauterine administration of ZK230211. Immunostaining for PR in endometrial stroma differed significantly ($P<0.05$) between the groups exposed to LNG and to ZK230211 at 4 μg/24 h.

Staining for ERα in both endometrial glands and stroma was similar following intrauterine exposure to LNG and all the three doses of ZK230211 Figs. 6.4C and 6.4D. In general, immunostaining for ERβ was more intense than for ERα. Immunolocalization ERβ in endometria following intrauterine treatment with ZK230211 and LNG is shown in Fig. 6.4E and F, respectively. Staining for ERβ was intense in surface epithelium, glands and stroma following exposure to both LNG and all three doses of ZK230211. No statistically significant differences in ERβ staining emerged between the endometria exposed to LNG and ZK230211.

Minimal staining for AR was detectable in the endometrial epithelium following exposure to intrauterine LNG. No immunoreactivity for AR was detectable in endometrial epithelium following ZK230211 ($P<0.05$ between groups exposed to LNG versus ZK230211). The endometrial stroma displayed moderate immunoreactivity for AR (NS between different subject groups) Figures 6.5A and 6.5B.

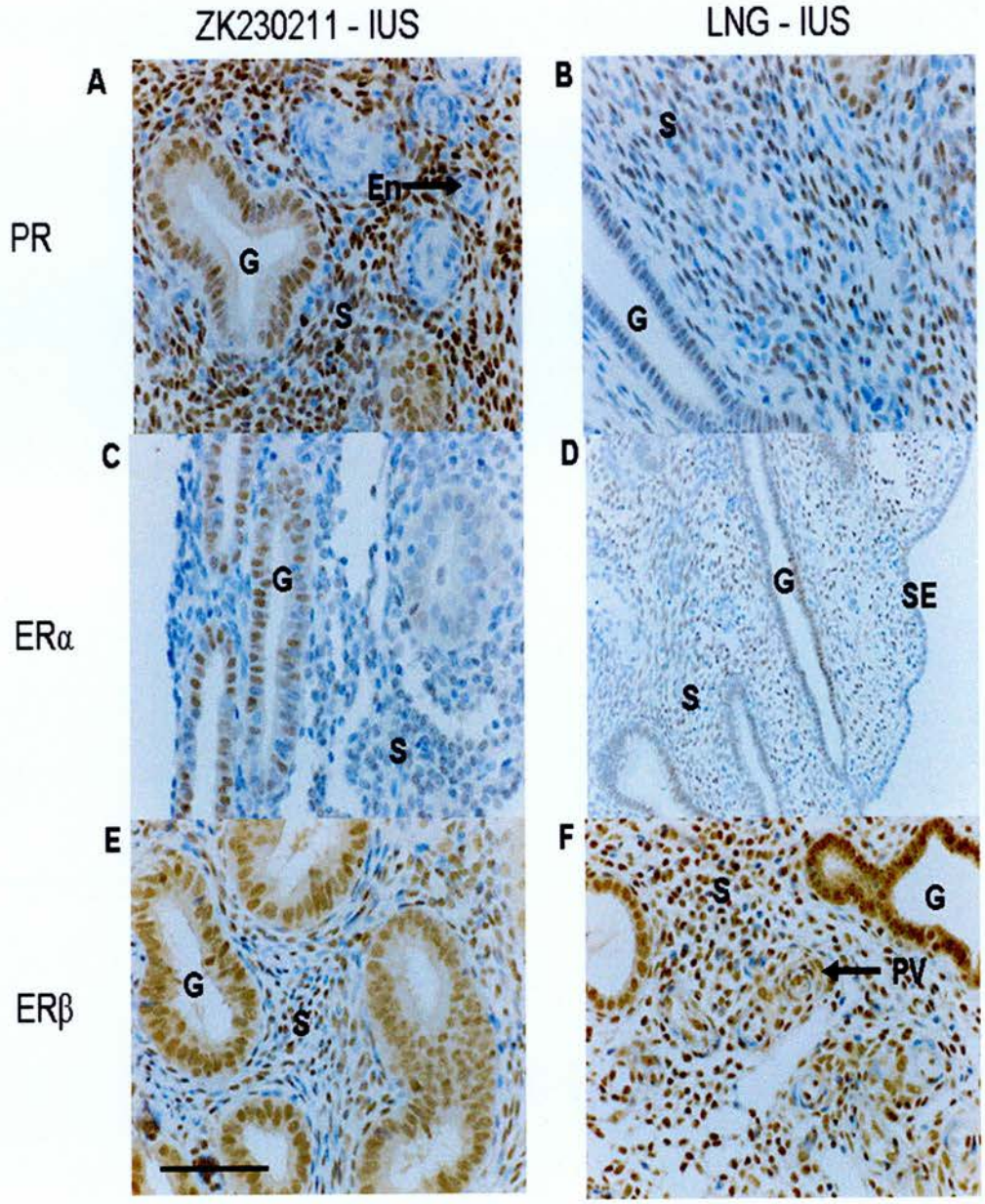
Proliferation marker expression

Tissue expression of the proliferation markers PH3 (Fig. 6.5C and D) and Ki-67 was weak in endometrial glands and stroma exposed to either ZK230211 or LNG. Staining for Ki-67 and PH3 was similar irrespective of the dose of ZK230211. When analysed semi quantitatively, the median values varied between 0 and 1, and did not differ between the different groups.

Decidualisation marker - IGFBP-1

Figures 6.5E and 6.5F depict the results of IGFBP-1 IHC in endometria following treatment with intrauterine ZK230211 and LNG, respectively. No immunostaining for IGFBP-1 was observed in endometria exposed to intrauterine ZK230211. However, IGFBP-1 was detectable in all but one of the endometrial specimens following intrauterine LNG administration ($P<0.0001$).

Figure 6.4 Immunohistochemical localization of PR, ERα and ERβ in human endometrium. (A) PR immunostaining in endometria from women treated with IUS's releasing 4 μg of ZK230211/24 h. Note the high level of stromal (S) immunoreactivity ($P < 0.05$) and increased immunostaining in glands (G). (B) PR immunostaining in endometria from women treated with LNG-IUS, demonstrating low levels of stromal and glandular PR immunoreactivity. ERα immunostaining in endometria from women treated with ZK230211 IUS releasing 4 μg of ZK230211/24 h (C). (D) ERα immunostaining in endometria from women treated with LNG-IUS. Staining for ERα was similar in endometrial glands and stroma after exposure to both, ZK230211 IUS and LNG-IUS. ERβ immunostaining in endometria from women treated with IUS's releasing 8 μg of ZK230211/24 h (E), and LNG-IUS (F). Note the high level of immunostaining at all cellular locations following use of both ZK- and LNG-IUS. Scale bar = 10 microns.
G = glands; S = stroma; SE = surface epithelium; En = endothelium; PV = perivascular cells


ZK230211 - IUS
LNG - IUS
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SE
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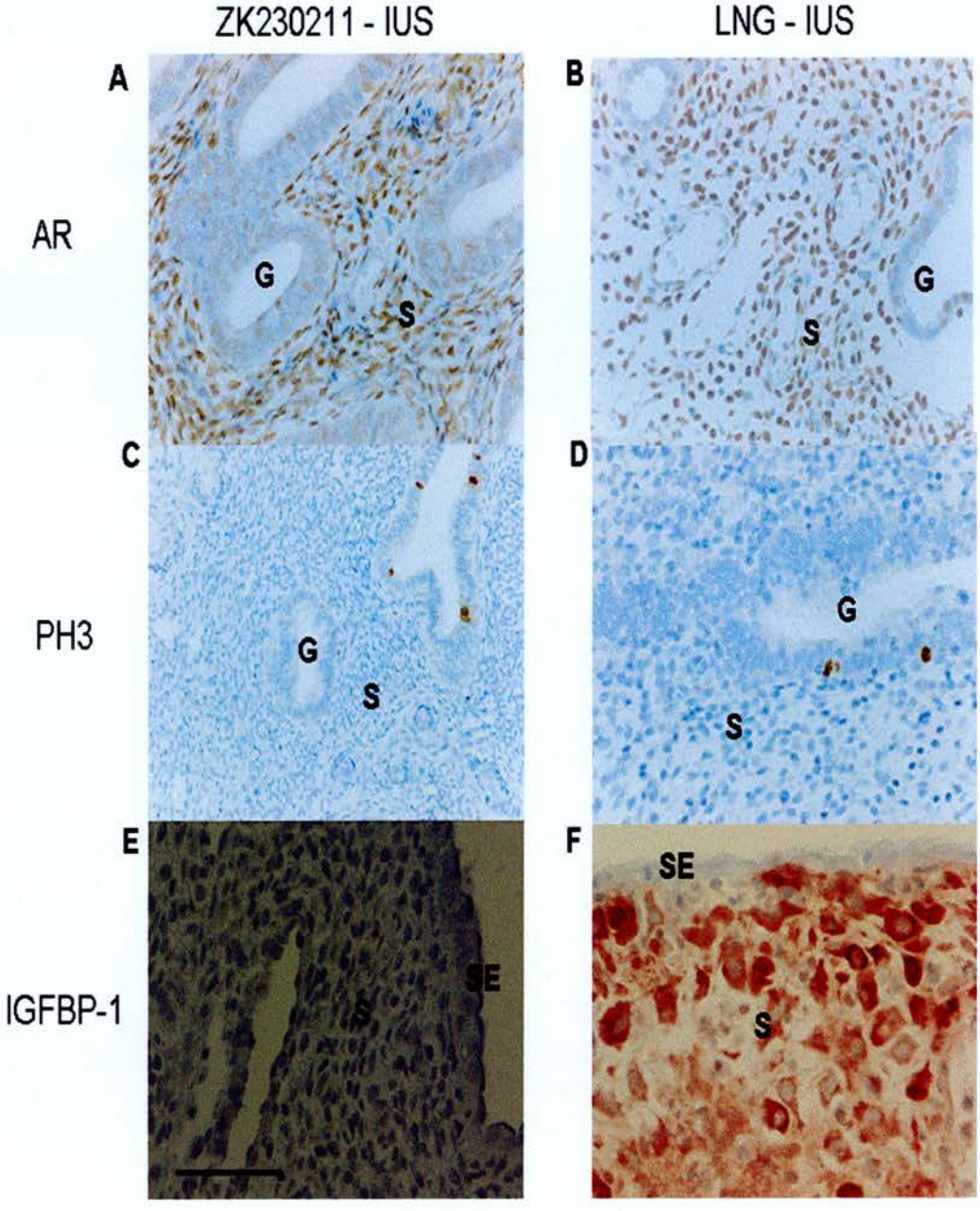
E
F
ERβ
G
S
S
G
PV

Figure 6.5 Immunohistochemical localization of AR, PH3 and IGFBP-1 in human endometrium. AR-stained endometrium following treatment with 8 μg of ZK230211/24 h (A), and treatment with an LNG-IUS (B). Note moderate staining in the stroma irrespective of the type of stimulation. PH3-stained endometrium following treatment with 4 μg of ZK230211/24 h (C), and treatment with an LNG-IUS (D). Note the sparse staining for PH3 and lack of proliferation. (E) IGFBP-1 immunostaining in endometria from women treated with an IUS releasing 8 μg of ZK230211/24 h. Note no immunoreactivity at any cellular location. (F) IGFBP-1 immunostaining in endometria from women treated with LNG-IUS. Note intense IGFBP-1 immunoreactivity in decidualized stromal cells. Scale bar = 10 microns
G = glands; S = stroma; SE = surface epithelium; En = endothelium


ZK230211 - IUS
LNG - IUS
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IGFBP-1
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6.4 Discussion

The present study demonstrates that intrauterine release of the PA ZK230211 by means of an IUS is feasible and that it results in significant endometrial levels of ZK230211. Use of a ZK-IUS had no effect on the number of days of bleeding or spotting, whereas an increase in uterine bleeding was noted during the short period of exposure of the endometrium to LNG. When compared with the LNG-IUS, clear signs of PA effects, such as maintenance of endometrial PR expression in the glandular epithelium and lack of IGFBP-1 protein expression, were seen during the short period of exposure to a ZK-IUS.

The present study was randomized and blinded in design. In addition, in contrast to the majority of studies in which the endometrial effects of PA have been assessed, the present work was performed in human subjects. A proof of concept study such as this, with obvious restrictions as regards clinical work, can only be performed among women presenting electively for hysterectomy. Thus a concern about interpretation of the data is that the IUS's were tested in women presenting with menstrual complaints and abnormal uteri. Furthermore, the study was of a relatively short duration and some of the eventual endometrial effects associated with both the LNG-IUS and ZK-IUS's may not be apparent until after a longer period of exposure. Analysis of the uterine blood vessels was not included in the present study. This is an interesting research question that should also be addressed in future studies. As expected, the number of days of spotting and bleeding increased following insertion of an LNG-IUS. However, the ZK-IUS's failed to have an effect on uterine bleeding patterns reported by the women. Since administration of PA to nonhuman primates results in a rapid induction of amenorrhoea (Wolf *et al.*, 1989; Slayden and Brenner, 1994; Slayden *et al.*, 1998; Slayden *et al.*, 2001), the lack of an immediate effect of the ZK-IUS's on uterine bleeding was an unexpected observation. In previous studies in which ZK230211 has been evaluated in non-human primates, ovulation and menstruation were suppressed in a dose-dependent fashion (Slayden *et al.*, 2001). The doses of ZK230211 used in the present work may have been insufficient for induction of amenorrhoea. However, in a preliminary study on non-human primates, intrauterine release of ZK230211 at 3–4 μg/24 h resulted in marked endometrial

suppression (Nayak *et al.*, 2000). The difference in bleeding patterns during the use of ZK-IUS's may be dose-related or the result of subtle differences between human and non-human primate endometrium.

Several investigators have reported profound atrophy of the endometrial glands and decidualization of stroma in women following use of an LNG-IUS for 3 months or longer (Silverberg *et al.*, 1986; Critchley *et al.*, 1998a; Phillips *et al.*, 2003). In the present study, the endometrium was categorized as suppressed in two-thirds of the LNG-IUS users. More profound suppression of the endometrium may have been observed had the duration of intrauterine LNG administration been for a longer period of time. Endometrial suppression, as evaluated morphologically, was weak following administration of ZK230211. In contrast, clear morphological signs of an estrogenic effect were evident in endometrial specimens collected following the use of intrauterine ZK230211. This is in agreement with the results of a recent study carried out by Baird et al. (Baird *et al.*, 2003), in which prolonged administration of low doses of oral mifepristone resulted in either a proliferative condition or cystic dilatation in the majority of women. Data concerning the morphological effects of PA, and more broadly those of selective PR modulators, on human endometrium have only recently been reported (Chwalisz *et al.*, 2005; Williams *et al.*, 2007). Categorization of the effect on the endometrium of ZK230211 is problematic, and the nomenclature associated with morphological events, such as estrogen or progestin-like, seen during the normal menstrual cycle, may not be justified. Indeed, it has been recognized that use of PR modulators leads to non-classical endometrial morphology, and new descriptions have been developed (Williams *et al.*, 2007).

In the present study, endometrial proliferation was assessed using IHC for both Ki-67 and PH3. The PH3 antibody identifies chromosomes during mitosis in cultured cells or whole mounts, thus providing accurate information about cellular proliferation (Brenner *et al.*, 2003). The immunoreactivity of these was negligible in both endometrial epithelial and stromal cell compartments following intrauterine delivery of LNG and ZK230211. Similarly, Hurskainen et al. (Hurskainen *et al.*, 2000) reported that endometrial staining for Ki-67 was weak during the use of an LNG-IUS. Previous studies concerning the effects of administration of PAs on

endometrial expression of Ki-67 have also been reported. Low levels of Ki-67 immunostaining were seen during administration of mifepristone at doses of 2 or 5 mg for 120 days in female volunteers (Baird *et al.*, 2003). In non-human primate endometrium, however, Ki-67 levels comparable to those seen in proliferative phase endometrium were detected following systemic administration of ZK230211 in cynomolgous monkeys undergoing artificial cycles (Slayden *et al.*, 2001). As regards PH3 immunolabelling, a significant decrease in the expression of PH3 has been reported in human endometrium following chronic low dose administration of mifepristone (Narvekar *et al.*, 2004).

Down-regulation of endometrial ER and PR is a well characterized progestin effect, and is observed with intrauterine administration of LNG (Critchley *et al.*, 1998b; Hurskainen *et al.*, 2000). In the present study, PR were down regulated both in the surface epithelium and in the stroma of the endometria exposed to LNG when compared with endometria exposed to ZK230211. As observed in nonhuman primate endometrium, endometrial PR levels were maintained during PA administration (Slayden *et al.*, 1993). The present data on PR expression in endometrium exposed to intrauterine PA are consistent with the PA nature of ZK230211. Endometrial expression of both ERα and ERβ was similar to the use of either an LNG-IUS or a ZK-IUS. This contrasts with the effects of ZK230211 on primate endometrium, in which systemic administration of ZK230211 counteracted the effects of progesterone, resulting in up-regulation of ERα (Slayden *et al.*, 2001).

Expression of AR is down regulated in secretory phase human endometrium and in endometria collected from women using an LNG-IUS (Burton *et al.*, 2003). In contrast, PA-induced suppression of endometrial growth is associated with up-regulation of endometrial AR in non-human primates (Brenner and Slayden, 2005) and in women (Narvekar *et al.*, 2004). Furthermore, antagonism of androgen action by co-administration of flutamide to PA-treated cynomolgous monkeys has been reported to result in loss of endometrial suppression (Brenner *et al.*, 2003). These data suggest that androgen action in essential for the suppressive effects of PAs on the endometrium. In the present study, expression of AR in the glandular epithelium was minimal among the subjects exposed to intrauterine ZK230211

administration. This lack of up-regulation of AR may explain in part the lack of endometrial suppression following intrauterine delivery of ZK230211.

The endometrial content of IGFBP-1 is increased in human secretory phase and decidualized endometrium (Rutanen *et al.*, 1984a; Rutanen *et al.*, 1984b). Similarly, addition of progestins to cultures of human endometrial tissue explants has been reported to increase the synthesis of IGFBP-1 (Bell *et al.*, 1991; Gao *et al.*, 1994). Endometrial exposure to LNG results in strong expression of endometrial IGFBP-1 (Pekonen *et al.*, 1992). Thus, sequestration of IGF action as a result of elevated tissue content of IGFBP-1 has been proposed as one of the mechanisms explaining endometrial suppression during use of an LNG-IUS (Rutanen *et al.*, 1997). In the present study, strong expression of IGFBP-1 protein was evident in the specimens collected from women treated with an LNG-IUS. However, IGFBP-1 was not detectable in any of the endometrial specimens collected from women using ZK-IUS's. It is possible that in the absence of IGFBP-1, IGF actions on the endometrium are maintained, which may contribute to the endometrial morphology observed during use of a ZK-IUS. Such data further confirm the PA action of ZK230211 on the human endometrium.

Conclusions

This study demonstrates that the effects of intrauterine release of the PA, ZK230211, and the progestin LNG, on the endometrium, differ. Endometrial proliferation is a function of mitotic activity in the endometrial epithelium. In general, progesterone antagonists, when administered chronically at relatively low doses, have been found to block mitotic activity of endometrial epithelium and induce stromal compaction thereby suppressing endometrial proliferation. However, such endometrial suppression following administration of ZK230211 was negligible. In the current study, ZK230211 was administered for a relatively short duration of time and this may be a contributory factor. Nonetheless, even after relatively short term use, lack of proliferative activity was evident in endometria from women exposed to ZK230211. A high level of endometrial PR expression as well as lack of IGFBP-1 protein expression following intrauterine administration of ZK230211 confirms the PA nature of ZK230211 action on the

human endometrium. Since the LNG-IUS has also been demonstrated to have lack of endometrial proliferative effects, it is likely that the clinical effects of intrauterine delivery of ZK230211 and LNG, on the human endometrium are probably mediated via different molecular and cellular mechanisms. The clinical potential of intrauterine delivery of PA should be evaluated in further clinical trials.

CHAPTER 7

General Discussion

7.1 General Discussion

This thesis is based on three studies predominantly evaluating endometrial steroid receptor expression after pharmacological exposure to three different but very pertinent clinical scenarios. It provides further clarification on endometrial characteristics at the time of embryo implantation in IVF/ICSI treatment cycles, during break-through bleeding in postmenopausal HRT treated endometrium and during treatment of premenopausal heavy menstrual bleeding. All studies were carried out on endometrium from human subjects.

The indications for assisted conception technologies are broadening. The treatment regimes commonly employ gonadotrophins, their analogues and antagonists to achieve various effects. In the context of IVF treatment, recent data show reduced pregnancy rates after treatment with a GnRH antagonist. The effects of GnRH antagonists could be mediated directly through the GnRH receptor, which has been localised in the endometrium or they may be mediated indirectly via the hormonal fluctuations that may lead to alteration in the expression of steroid receptors or indeed through an alteration in the intracrine modulation thereby affecting ligand availability within the endometrial cell.

In the first study included in this thesis, it is demonstrated that, in endometrium exposed to Gonal-F® (a recombinant – FSH preparation) and cetrorelix (a GnRH antagonist), significant down-regulation of PR protein expression occurs in the endometrial stroma and surface epithelium (Vani *et al.*, 2007). PR down-regulation in the surface epithelium is thought to be under the influence of progesterone and PR is known to decline at the beginning of the window of implantation. It therefore appears that any hormonal fluctuations that may result in the serum due to treatment with cetrorelix and Gonal-F® have little impact on intracellular PR in surface epithelium. In the secretory phase of a normal menstrual cycle, significant PR expression has been detected in the endometrial stroma. Several genes have been localized to the endometrial stroma and are expressed during the secretory phase. Studies have also shown an important role for progesterone in the endometrial stromal cell in induction of extracellular matrix in relation to implantation. A significant down-regulation of stromal PR, as observed after exposure to rFSH and a GnRH antagonist, could thus influence gene expression

during the secretory phase or indeed have an influence on the modulation of extracellular matrix.

After exposure to cetrorelix and Gonal-F®, PR was significantly up-regulated in the perivascular cells (Vani *et al.*, 2007). PR protein has not been identified in the vascular endothelium, but they are abundantly expressed in the perivascular cells throughout the menstrual cycle. Vascular proliferation and restructuring are important functions of secretory phase endometrium and progesterone acting on the perivascular cells has been implicated in the modulation of endometrial blood flow. Furthermore, cytokine control in the perivascular cells is thought to be controlled by progesterone.

Thus it is evident that there are significant alterations in PR expression after exposure to a GnRH antagonist and rFSH. As described above, there are a variety of possible mechanisms through which these factors could impact on the processes of embryo implantation and on pregnancy rates in IVF cycles. No significant differences were observed in the protein expression of ERα, ERβ, AR, 3βHSD or 17βHSD5 (Vani *et al.*, 2007).

Having established the PR protein alterations under the effect of rFSH and GnRH antagonist, it would be interesting to evaluate whether this translated into transcriptional changes. Hence quantitative PCR studies were undertaken. The results were disparate. Quantitatively, there was a significant reduction in the levels of ERα mRNA and AR mRNA but no changes in the ERβ mRNA transcripts. PR mRNA was reduced after treatment but the difference was not significant (Vani *et al.*, 2007). PR mRNA levels are known to vary during the menstrual cycle. The reason for the discrepancy between protein expression and mRNA expression is unclear but this phenomenon has been reported by other researchers. Changes in PR in whole biopsies may not reflect the subtle changes in steroid receptor expression that exist between individual cell types. Regarding ER, despite controlled ovarian hyperstimulation that is known to lead to supraphysiological levels of E2 and progesterone, no significant differences were observed in the immunoexpression of ERα or ERβ in treated women (Vani *et al.*, 2007). Even ER are thought to affect the endometrial receptivity through the predominantly progestational effects of endometrial phase advancement and premature

luteinization. Hence, it appears that ER-mediated signalling appears to be of less importance in relation to implantation compared with PR mediated effects on the endometrium. It is interesting to note however that quantitative RT–PCR showed significantly reduced ERα mRNA levels in the treatment group (Vani *et al.*, 2007).

AR is expressed in the endometrial stromal cells. The intensity of expression declines from proliferative phase to mid-secretory phase. No significant differences in protein expression of AR were observed in women treated with rFSH and a GnRH antagonist. However, QRT–PCR showed significantly reduced AR mRNA levels in the group of women treated with a GnRH antagonist and rFSH (Vani *et al.*, 2007). So far, there are very limited data on effects of AR and AR-induced gene expression in humans. Studies in pigs show AR in the pig endometrium during the window of implantation and demonstrate the functional, albeit complex, interactions of androgens and estrogens in the regulation of uterine endometrial gene expression and cell growth in vitro. Further studies are needed to evaluate AR-induced gene expression in humans and the potential impact on embryo implantation.

In summary, the protein and mRNA expression data thus far appear to show that the endometrial effects are mediated through progesterone acting on the stromal and peri-vascular PR with little influence of ER mediated signalling.

Having gathered data regarding the receptor expression and mRNA transcript profile, it becomes important to understand the intracellular changes in ligand availability. Endometrial intracrinology was studied by evaluating the 17β hydroxysteroid dehydrogenases and the 3β hydroxysteroid dehydrogenase enzymes, well recognised members in the intracrine cascade. In this study, irrespective of presence or absence of treatment, no significant differences were observed in endometrial 3βHSD immunoexpression between the two groups of women (Vani *et al.*, 2007). This suggests that the pre-ovulatory supraphysiological levels of estrogen and progestogen that result from COH and use of GnRH antagonists do not lead to any significant alteration in the levels of 3βHSD protein during the window of implantation. The currently available antibody against 3βHSD recognizes both forms of human 3βHSD enzymes, types 1 and 2. Hence it is not possible to comment on changes in the amounts of 3βHSD1 protein.

However, RNA studies indicate that 3βHSD1 mRNA transcripts may be changing. A significant reduction in 3βHSD1 mRNA was observed in women treated with a GnRH antagonist and rFSH (Vani *et al.*, 2007). The 3βHSD is responsible for the conversion of inactive pregnenolone to active progesterone and of dehydroepiandrosterone to androstenedione. A reduction in 3βHSD1 transcripts will ultimately lead to a reduction of intracellular progesterone. In the presence of altered PR expression, as was observed with IHC, it is likely that the reduced ligand availability for binding to PR leads to a disturbance in the dynamics of ligand-receptor interaction. This may affect progesterone mediated signalling pathways including alterations in gene expression profiles thereby affecting the receptivity of the endometrium.

The 17βHSD enzymes are involved in the reactions catalyzing the activation and inactivation of androgens and estrogens. The 17βHSD5 transforms androstenedione to testosterone and also progesterone to the inactive 20-hydroxyprogesterone. The 17βHSD2 is involved in the inactivation of E2 to estrone and converting androgens to less potent forms. A significant reduction of 17βHSD2 mRNA was observed in women treated with rFSH and a GnRH antagonist (Vani *et al.*, 2007). Since 17βHSD2 is involved in the inactivation of E2 to estrone and converting androgens to less potent forms, it is likely that higher levels of intracellular E2 and androgens persist thereby further disturbing the balance between estrogen, progesterone and androgens. This may further affect the endometrial development leading to suboptimal endometrial receptivity.

The first study included in this thesis has addressed one of the vital functions of the endometrium, that of embryo implantation. Another important aspect of endometrial function is its response to postmenopausal hormone therapy and this has been a matter of great interest in the last several decades. Postmenopausal hormone replacement therapy is widely used. However, breakthrough bleeding (BTB) on HRT, causes of which are unknown, is a common problem in all HRT users. It is recognised that altered endometrial blood vessels, MMP's and their tissue inhibitors, changes in endometrial leucocyte populations and uterine natural killer cells have a role in HRT related BTB. However the exact role of steroid receptors in BTB is unclear. A detailed description of steroid receptors; PR, GR,

AR, ERα and ERβ receptor protein expression in endometrial cell components (glands, stroma, surface epithelium, perivascular and endothelial cells) of postmenopausal HRT users is vital to understand the causes of BTB and to develop treatment strategies to prevent this unwanted side effect. In the second study included in this thesis, steroid receptor expression has been described in the context of bleeding patterns reported by subjects, with a particular advantage of inclusion of endometrial biopsies obtained at the time of a bleeding episode.

All HRT users in the said study were using continuous combined HRT, which has a predominantly progestogenic effect on the endometrium. Hence one would expect to see progestational change in the endometrium, likely mimicking the secretory phase of a normal menstrual cycle or changes similar to those seen in women using progestogen only contraception. The endogenous and exogenous hormonal milieu in postmenopausal HRT users is, however, quite different from that seen in the secretory phase in premenopausal women where the specific sequential pattern of ovarian steroid production is tightly regulated. Significant differences in steroid receptor expression have not been observed in endometrium of women using HRT who report unscheduled bleeding episodes. Interesting trends (non-significant) were observed in endometrial expression of glandular PR (decrease) and GR (increase) in postmenopausal HRT users who report unscheduled bleeding. There were no differences in AR or ER (α and β) expression, irrespective of presence or absence of HRT and bleeding pattern (Vani *et al.*, 2008). These patterns of steroid receptor expression in HRT users (with and without unscheduled bleeding) differ from those seen in premenopausal women using progestogen-only contraception, with bleeding from an apparently 'atrophic' endometrium. Absence of significant differences assumes a special importance not least due to the dissimilarities noted between what is expected and what was observed. Hence it seems likely that different mechanisms underlie abnormal bleeding in postmenopausal HRT users.

An interesting, albeit non-significant, observation was that with HRT, strong immunoexpression of PR in endometrial glands was maintained but in the presence of reported unscheduled bleeding glandular PR expression declined. Unlike the premenopausal state, in the postmenopausal untreated endometrium, strong glandular PR expression is maintained (Vani *et al.*, 2008). In regular cycling

women, PR is down-regulated in the glandular compartment during the secretory phase by progesterone.

Another interesting observation was the absence of PR expression in the perivascular cells in all studied biopsies. No perivascular PR expression was observed irrespective of the presence or absence of HRT and with or without bleeding (Vani *et al.*, 2008). This observation appears to be a feature of postmenopausal endometrium in contrast to premenopausal endometrium since, in the premenopausal state, at least in women with regular cycles, progesterone receptors are abundantly expressed in the perivascular endometrial cells throughout the cycle.

Inflammation has been proposed as a possible contributory factor in BTB with or without exposure to HRT. With this in mind, GR expression was analysed in the postmenopausal endometrium. Endometrium GR is normally localised to stromal and vascular endothelial cells. Increased GR expression may potentiate effects of endogenous/locally derived cortisol thus suppressing angiogenesis and aggravating potential for unscheduled bleeding. Augmented local cortisol action may also disturb local endometrial prostaglandin production. Disturbance in the balance of local factors maintaining endometrial blood vessel integrity and stability could be another possible mechanism responsible for unscheduled bleeding.

In the study included in this thesis, five different preparations of continuous combined HRT preparations were used. This may have contributed to the differences in these findings. Nevertheless, irregular bleeding is common to all HRT preparations and hence it is reasonable to work on the assumption that common mechanisms may underlie these phenomena. Alternatively it may suggest variable functional alterations of ER and PR in the postmenopausal endometrium during exposure to differing hormonal preparations.

Having dealt with the questions of steroid receptor expression in mid-luteal endometrium in the context of embryo implantation and then postmenopausal endometrium in the context of BTB, attention was directed to another vital endometrial function; menstruation. Menstrual dysfunction is one of the commonest complaints in gynaecology. Progestogens are commonly used for the treatment of menstrual disorders but all progestogens have a common side effect –

breakthrough vaginal bleeding. The levonorgestrel –IUS is no exception to this annoying side effect and a significant proportion of users have the IUS removed as a result of this problem. Progesterone antagonists (PA) have been proposed as alternatives since, in animal studies, use of PA-IUS has resulted in complete amenorrhoea with endometrial suppression without suppressing ovarian hormonal activity.

The third study included in the thesis is a proof of concept study. Here, expression of sex steroid receptors PR, ERα, ERβ and AR; proliferation markers PH3 and Ki-67 and decidualisation marker IGFBP-1 was evaluated. Intrauterine release of the PA ZK230211 appears to be feasible and it results in significant endometrial levels of ZK230211. When compared with the LNG-IUS, clear signs of PA effects, such as maintenance of endometrial PR expression in the glandular epithelium and lack of IGFBP-1 protein expression, were seen during the short period of exposure to a ZK-IUS (Heikinheimo *et al.*, 2007).

As expected, the number of days of spotting and bleeding increased following insertion of an LNG-IUS. However, the ZK-IUS's failed to have an effect on uterine bleeding patterns reported by the women (Heikinheimo *et al.*, 2007). Since administration of PA to nonhuman primates results in a rapid induction of amenorrhoea, the lack of an immediate effect of the ZK-IUS's on uterine bleeding was an unexpected observation. The doses of ZK230211 used in the present work may have been insufficient for induction of amenorrhoea. The difference in bleeding patterns during the use of ZK-IUS's may be dose-related or the result of subtle differences between human and non-human primate endometrium.

In relation of steroid receptors, down-regulation of endometrial ER and PR is a well characterized progestin effect and is observed with intrauterine administration of LNG. In the present study, PR were down regulated both in the surface epithelium and in the stroma of the endometria exposed to LNG when compared with endometria exposed to ZK230211 (Heikinheimo *et al.*, 2007). The present data on PR expression in endometrium exposed to intrauterine PA are consistent with the PA nature of ZK230211. Endometrial expression of both ERα and ERβ was similar to the use of either an LNG-IUS or a ZK-IUS (Heikinheimo *et al.*, 2007). This contrasts with the effects of ZK230211 on primate endometrium, in

which systemic administration of ZK230211 counteracted the effects of progesterone, resulting in up-regulation of ERα.

Previous studies assessing AR expression suggest that androgen action is essential for the suppressive effects of PAs on the endometrium. In the present study, expression of AR in the glandular epithelium was minimal among the subjects exposed to intrauterine ZK230211 administration (Heikinheimo *et al.*, 2007). This lack of up-regulation of AR may explain in part the lack of endometrial suppression following intrauterine delivery of ZK230211.

Endometrial proliferation was assessed using IHC for both Ki-67 and PH3. The immunoreactivity of these was negligible in both endometrial epithelial and stromal cell compartments following intrauterine delivery of LNG and ZK230211 (Heikinheimo *et al.*, 2007). Previous studies in women have shown low levels of Ki-67 immunostaining during administration of mifepristone however in non-human primate endometrium, Ki-67 levels comparable to those seen in proliferative phase endometrium were detected following systemic administration of ZK230211. As regards PH3 immunolabelling, a significant decrease in the expression of PH3 has been reported in human endometrium following chronic low dose administration of mifepristone.

The endometrial content of IGFBP-1 is increased in human secretory phase and decidualized endometrium. Endometrial exposure to LNG results in strong expression of endometrial IGFBP-1. Thus, sequestration of IGF action as a result of elevated tissue content of IGFBP-1 has been proposed as one of the mechanisms explaining endometrial suppression during use of an LNG-IUS. In the present study, strong expression of IGFBP-1 protein was evident in the specimens collected from women treated with an LNG-IUS. However, IGFBP-1 was not detectable in any of the endometrial specimens collected from women using ZK-IUS (Heikinheimo *et al.*, 2007). It is possible that in the absence of IGFBP-1, IGF actions on the endometrium are maintained, which may contribute to the endometrial morphology observed during use of a ZK-IUS. Such data further confirm the PA action of ZK230211 on the human endometrium.

Conclusions:

This thesis adds to our understanding of sex steroid receptor expression in relation to three important clinical areas pertaining to the endometrium i.e. embryo implantation, menstrual bleeding and postmenopausal hormone therapy related breakthrough bleeding. All these clinical scenarios have, at the biological level, involvement of sex steroid receptors and other factors in a complex cascade of events. The above studies show that whilst steroid receptors have a role to play in each situation, the interactions are complex. They also suggest that receptor activity and effects may be species specific, may vary with age and the nature and duration of pharmacological stimulus. To understand the functional significance of the steroid receptors, specific questions will need to be addressed in focussed studies. With further clarity of endometrial steroid receptor function, there is potential for developing clinically effective interventions.

7.2 Suggestions for future studies

This thesis encompasses 3 studies analysing the endometrium under the effects of different pharmacological interventions. These are in relation to 3 clinical scenarios however through these studies the hope was to gain further understanding of the modulation of the local endometrial steroid milieu.

Endometrium in embryo implantation

In comparison to physiological mid-luteal endometrium, it has been demonstrated that with exogenous administration of a GnRH antagonist and recombinant FSH, there are alterations in the sex steroid receptor expression and in the intracellular expression of steroid metabolizing enzymes. These changes may lead to alterations in the activity and the intracellular availability of the ligands.

- This sample size in this study was small and in the first instance it would be interesting to undertake a similar study on larger number of women to confirm these findings.
- Alterations in the steroid receptor expression have been observed and it would be interesting to see if these led to morphometric changes in the various cellular compartments in the mid-luteal endometrium.
- In the recent past, in relation to embryo implantation, the focus of research has shifted to gene array studies. It is recognised that sex steroids, predominantly acting on the endometrial stromal cells, have a significant influence on the gene profiles and an alteration of the ligand availability may have a significant impact on the gene profile in the mid-luteal phase. Gene micro-array studies would be useful to assess the impact of rFSH and GnRH antagonist exposure on mid-luteal endometrium.
- Ultimately it would be interesting to analyse the impact of these observations on embryo implantation and pregnancy rates in IVF cycles

Endometrium and post-menopausal hormone replacement therapy

Hormone replacement therapy users frequently suffer from unscheduled breakthrough bleeding. The mechanisms are unclear. Interesting trends, albeit non-significant in this small study, have been demonstrated in endometrial expression of glandular PR and GR in postmenopausal HRT users who report unscheduled bleeding. The steroid receptor expression observed in women on continuous

combined HRT was dissimilar from women on long-term progestins. Hence it appears that different mechanisms apply in HRT related unscheduled bleeding.

- The observed trends in the GR expression raise interesting questions about a possible mechanism related to the inflammation pathway. If indeed the GR expression findings are confirmed in a larger study then further studies should be directed to evaluating the inflammatory markers in the postmenopausal endometrium.
- Another interesting observation was the downward trend of PR in glands. It is unclear whether this was a cause or effect of the breakthrough bleeding. Quantitative evaluation of PR mRNA and intracrine studies might clarify whether this observation is due to a reduction in transcription or as a result of a fall in ligand availability.
- A very interesting feature in all postmenopausal endometrial samples was the absence of PR expression in the perivascular cells. This raises interesting questions about the vascular function of the putative 'atrophic' endometrium.

Endometrium and Anti-Progestin Intrauterine System

This study has demonstrated that intrauterine release of ZK230211 is feasible.

- Perhaps due to the short time duration of the study, the use of ZK-IUS did not show any effects on the number of days of bleeding or spotting. Hence in the first instance, studies need to be carried out over a longer period of time.
- In non-human primate studies, ZK-IUS, even at the doses used in this study, showed a rapid induction of amenorrhoea. This was not seen in the current study and one of the possible explanations is that the dose required to achieve amenorrhoea in humans may be different. Having established the feasibility of the use of a ZK-IUS, further studies need to identify the optimum effective dose of the ZK-IUS in humans to achieve satisfactory suppression of ovulation and menstruation without leading to a hypo-estrogenic state.

- In relation to the bleeding, analysis of the uterine blood vessels was not included in the present study. This is an interesting research question that should be addressed in future studies.
- Finally, trials need to be conducted to evaluate the clinical potential of the ZK-IUS.

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Appendix I: Source of General Materials

Tissue Collection	Supplier
Neutral Buffered Formalin (NBF)	See Appendix: Recipes
Paraformaldehyde (PFA)	See Appendix: Recipes
Pipelle Suction Curette	Laboratoire CCD, Paris, France
TP1050 Tissue Processor	Leica Corp., UK

RNA Extraction	Supplier
Trizol Reagent	Invitrogen Life Tech, Paisley, UK
Hand Held Homogeniser (Polytron PT 1200B)	Kinematica, Switzerland
Chloroform	Sigma, Poole, Dorset, UK
Isopropranolol	Sigma, Poole, Dorset, UK
Ethanol (Molecular Biology Grade)	BDH Laboratory Supplies, Poole, UK
RNA Storage Solution	Ambion Inc., Austin, Texas, USA
Phase Lock Gel Tubes	Eppendorf, Hamburg, Germany

Real Time QRT-PCR	Supplier
DNAse Amplification Kit	Invitrogen Life Tech, Paisley, UK
Taqman Reverse Transcription Reagents	AB Applied Biosystems, Cheshire, UK
Taqman Master Mix	Stratagene, Amsterdam, Netherlands
Ribosomal RNA Control Reagents	AB Applied Biosystems, Cheshire, UK
Primers/ Probe Sets for Sequences	Biosource, Nivelles, Belgium
ABI Prism 7700	AB Applied Biosystems, Cheshire, UK

Immunohistochemistry	Supplier
Histoclear	Nat. Diagnostics, Atlanta, Georgia, USA
Phosphate Buffered Saline (PBS)	See Appendix II
PBS+Tween 20	See Appendix II
Tris Buffered Saline (TBS)	See Appendix II
TBS+Tween 20	See Appendix II
Hydrogen Peroxide Solution 30%	BDH Laboratory Supplies, Poole, UK
Sodium Citrate	See Appendix II
Pressure Cooker	Clipso, Tefal, Nottingham, UK
Avidin/Biotin Blocking Kit	Vector Laboratories, Peterborough, UK
Non Immune Horse Serum (Vectastatin)	Vector Laboratories, Peterborough, UK
Bovine Serum Albumin	Sigma, Poole, UK
Biotinylated Horse anti-Mouse Antibody	Vector Laboratories, Peterborough, UK
Avidin Biotin Peroxidase Complex	Vector Laboratories, Peterborough, UK
3,3' Diaminobenzidine	Dako, Cambridge, UK
Harris Haematoxylin	Pioneer Research Chemicals, Colchester, UK
Xylene	BDH Laboratory Supplies, Poole, UK
Pertex	Cellpath Plc, Hemel Hempstead, UK
Image Analysis System	Improvision Inc., Lexington, MA, USA

Appendix II: Recipes for Solutions

4% Paraformaldehyde (PFA)		
Weight/Volume	**Chemical Name**	**Supplier**
4g	Paraformaldehyde	Sigma
100ml	PBS (See Below)	Sigma
Heat at 60^0C Store at 4^0C and use within 7 days		

4% Natural Buffered Formalin (NBF)		
Weight/Volume	**Chemical Name**	**Supplier**
6.5g	Na_2HPO4	BDH
4.5g	$Na_2HPO4.2H2O$	BDH
100ml	40% Formaldehyde	BDH
900ml	Distilled Water	

0.01M Phosphate Buffered Saline (PBS) pH 7.4		
Weight/Volume	**Chemical Name**	**Supplier**
5 Tablets	PBS	Sigma
1000ml	Distilled Water	

0.01M Phosphate Buffered Saline+Tween (PBST) pH 7.4		
Weight/Volume	**Chemical Name**	**Supplier**
5 Tablets	PBS	Sigma
1000ml	Distilled Water	
8g	NaCl	BDH
100µl	Tween 20	Sigma

0.5M Tris Buffered Saline (TBS)		
Weight/Volume	**Chemical Name**	**Supplier**
60.55g	Trizma Base	Sigma
700ml	Distilled Water	
pH to 7.4 and make up to 1000ml with Distilled Water Dilute 1:10 for working 0.05 Solution and add 8.5g NaCl/litre		

0.5M Tris Buffered Saline+Tween 20 (TBST)		
Weight/Volume	**Chemical Name**	**Supplier**
60.55g	Trizma Base	Sigma
700ml	Distilled Water	
pH to 7.4 and make up to 1000ml with Distilled Water Dilute 1:10 for working 0.05 Solution and add 8.5g NaCl/litre Add 100µl/L Tween 20		

0.1M Sodium Citrate		
Weight/Volume	**Chemical Name**	**Supplier**
29.41g	Tri-Sodium Citrate	BDH
0.1g	Sodium Azide	Sigma
700ml	Distilled Water	
pH to 6.0 and make up to 1000ml with Distilled Water Dilute 1:10 for working 0.01M Solution		